THE EFFECT OF KELP SUPPLEMENTATION IN FORMULATED FEED ON THE PRODUCTION PERFORMANCE AND GUT MICROBIOTA OF SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*)

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ABSTRACT

Formulated feeds with a relatively low (< 5 % of dry mass) kelp (*Ecklonia maxima*) inclusion level are widely used on commercial abalone (*Haliotis midae*) farms in South Africa. Although the use of kelp, a major constituent of the natural diet of *H. midae,* as a dietary supplement is considered to enhance abalone growth and feed utilisation, there are no published studies which quantify the effects of kelp inclusion in formulated feeds. Furthermore, the physiological mechanisms by which kelp supplementation may positively influence abalone digestive physiology and growth are largely unknown. As the kelp supplement is comprised mostly of soluble fibres and abalone gut bacteria associated with macroalgae (and its fibrous polysaccharides) are known to play a key role in digestion, it was hypothesised that the kelp supplement influences the gut-bacterial community profiles of cultured abalone through prebiotic and other metabolic effects. The present thesis thus examined the effect of kelp supplementation on the performance of abalone (*Haliotis midae*) fed formulated feeds and explored the influence of a kelp supplement on the abalone gut microbiota and its function in the gastrointestinal tract.

The key hypotheses of the study were that kelp supplementation in formulated feed: 1) enhances abalone growth; 2) causes a shift in abalone gut-bacterial community composition through a prebiotic-like effect; 3) may induce changes in crop morphology as a result of potential bacterial-associated increases in volatile short-chain fatty acids, and 4) alters digestive enzyme activities in the abalone gut through changes in bacterial-derived (exogenous) digestive enzymes.

The growth-promoting efficacy of low-level kelp supplementation was tested by feeding isonitrogenous and isoenergetic experimental feeds containing 0.00 – 3.54 % kelp (dry mass) to sub-adult abalone (~43 mm shell length) for eight months under commercial

farm conditions. The growth trial established that kelp supplementation (0.44 – 3.54 % of dry mass) promoted faster growth and improved feed conversion and protein efficiency ratios in cultured abalone compared to abalone fed the non-supplemented control diet, while there were no significant differences in growth for abalone fed the different kelp-supplemented diets (0.44, 0.88, 1.76 and 3.54 % of dry mass). Feed conversion and protein efficiency ratios displayed significant correlations with kelp level in the range of 0.00 – 3.54 % dry mass, and it is therefore recommended that kelp be included in the formulated feeds of cultured South African abalone at a rate of up to 3.54 % of dry mass.

A kelp-supplemented (0.88 % dry weight inclusion) feed was fed to abalone under farm conditions to compare gut physiological parameters (crop morphology, digestive enzyme activities and the gut microbiota) in abalone against that of abalone fed an isonitrogenous and isoenergetic non-supplemented control feed.

To establish if the observed higher abalone growth rates were related to improved gastrointestinal tract epithelium activity and integrity, as reflected by epithelial cell growth in response to potential changes in bacterial-derived short-chain fatty acid production, crop epithelial morphology was compared between abalone fed the kelp-supplemented and control feeds. Kelp supplementation did not induce any observable changes in crop epithelial cell height for farm-reared sub-adult abalone fed the experimental diets on-farm for seven weeks. This was attributed to the similar macronutrient compositions of kelp-supplemented and control diets and/ or the common diet history of experimental abalone from weaning to the initiation of the experiment.

Digestive enzyme activity was compared between abalone fed a kelp-supplemented and a control feed during an on-farm feeding trial with sub-adult abalone. Gut samples were collected after seven weeks and colorimetric enzyme assays were performed for the polysaccharide-degrading enzymes amylase, alginate lyase, laminarinase and fucoidanase,

and for acid protease, trypsin and chymotrypsin activity. Amylase and alginate lyase activities were relatively high, compared to the other enzymes. Polysaccharidase and acid protease activity levels did not differ significantly between abalone fed kelp-supplemented and control feeds, but a greater variability in enzyme activity levels was observed in abalone fed the control diet. It was hypothesised that this might be due to the kelp supplement promoting a more stable and less opportunistic gut-bacterial community than the control diet.

Pooled gut samples of abalone fed the kelp-supplemented diet were used for proteomic analyses to identify the composition of enzyme proteins of both endogenous and exogenous origin in the abalone digestive system. The key polysaccharidases and proteases in the gut samples of kelp-supplemented formulated feed-fed abalone were all of abalone origin, whereas the bacterial enzymes were of the types that form part of intermediate reactions in metabolic pathways. The results suggested that bacterial enzymes play a different role to abalone endogenous enzymes in the digestion of formulated feed. While abalone enzymes appear to be the main degraders of carbohydrate and protein macromolecules, the profile of exogenous enzymes suggests that they perform bioconversions of smaller organic compounds.

The profiles of gut-bacterial communities of farm-reared sub-adult abalone fed kelp-supplemented and control feeds on-farm for seven weeks were analysed with metagenomic pyrosequencing and DGGE analyses, using 16S rDNA-targeted amplified DNA. The results indicated a shift in gut-bacterial composition with a higher abundance of Mollicutes in abalone fed kelp-supplemented feed compared to those fed the control feed. DGGE band patterns displayed a greater within-group similarity in gut bacteria for abalone fed the kelp-supplemented diet and the presence of unique and variable bands for bacteria in the guts of abalone fed the control diet. It was concluded that when cultured abalone are fed kelp-supplemented formulated feeds, more stable gut bacterial communities are present compared

to a more opportunistic gut-bacterial community in abalone fed non-supplemented feeds, and that the observed increase in Mollicutes could reflect the restoration of the abalone gut microbiota to a more natural state.

The novel application of proteomics to abalone nutrition in the present study demonstrated that gut-bacterial enzymes may form part of many different metabolic pathways and suggests that the metabolism of the gut microbiota serves as an extension of the abalone's digestive metabolism. Future studies should quantify the contribution of commensal gut-bacteria to cultured abalone nutrition by employing metabolomic studies to characterize the utilisation of bacterial-derived metabolites by the abalone host.

DECLARATION

I certify that this work is my own and that, to the best of my knowledge, it does not incorporate any information published or written by others without acknowledgment and referencing.

Aldi Nel

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Seaweed has a low macronutrient and protein content and yet it is the preferred and natural food source of abalone. The digestive physiology of abalone by which they efficiently utilise seaweed to obtain balanced nutrition remains unknown. Formulated nutrient-rich feeds that are based on the formulation principals of conventional feeds used for monogastric animal nutrition have been successfully employed in the culture of different abalone species. Most of the *Haliotis midae* abalone that are farmed locally are produced using formulated feeds, but abalone have to be introduced to the feeds at a relatively early developmental stage to display good production performance on formulated feeds (Knauer et al. 1996). A growing body of literature suggests that the use of seaweed in addition to formulated feed in abalone farming improves feeding activity, feed utilisation, growth, health and marketability of farmed abalone (Bansemer et al. 2014). Therefore, there is renewed interest in providing seaweed as a food source for cultured abalone, as part of a dietary regime or as a supplement in formulated feed.

Seaweeds are known to have a rich mineral and bioactive compound content (Mabeau & Fleurence 1993) in addition to its high content of complex polysaccharides with potential prebiotic properties (Erasmus et al. 1997; Sawabe et al. 2003). The use of seaweed on abalone farms becomes especially beneficial when abalone are subjected to warm water temperatures and physiological stress, during which dietary fresh seaweed can promote the antioxidant status, antiviral activity and survival of farmed abalone (Dang et al. 2011; Lange et al. 2014; Stone et al. 2014). Less is understood about the use of dried macroalgae in the formulated feeds of cultured abalone, and only one published study have investigated the use

of macroalgae meal in abalone feed and found that it can improve abalone feed utilisation efficiency (O'Mahoney et al. 2014).

There is published evidence of favourable influences on growth in locally-farmed *Haliotis midae* when abalone are fed fresh seaweed-Abfeed® combination diets (Dlaza et al. 2008), while fresh seaweed may stimulate feeding activity and improve feed utilisation in *H. midae* (Justin Kemp, unpublished data). Therefore, to capitalise on the performance benefits of dietary seaweed inclusion for farmed abalone, while also employing the convenience of water-stable formulated feeds, Abfeed®-K26 which includes the kelp (*Ecklonia maxima*) at a proprietary and relatively low inclusion level, has been one of the most widely used feeds on local abalone farms.

This chapter reviews the use of formulated feed and seaweed supplementation in the diets of cultured abalone and its potential effect on the abalone's digestive physiology and gut microbiota, and motivates the study's research approach and main objectives.

LITERATURE REVIEW OF FORMULATED FEEDS AND SEAWEED SUPPLEMENTATION AND ITS EFFECTS ON THE ABALONE GUT ENVIRONMENT AND MICROBIOTA

The abalone digestive system

South African abalone, *Haliotis midae*, feed opportunistically on drift macroalgae associated with their habitat (Barkai & Griffiths 1986). In the Western Cape Province, where extensive kelp-beds, wild abalone populations and most abalone farms are located, abalone feed predominantly on brown macroalgae. Following ingestion, there appears to be a continuous one-directional flow of food through the digestive tract, apart from selective processing of food slurry within the caecum and digestive gland (Campbell 1965). However, within the crop, which is the digestive tract's largest luminal area and a site for extracellular digestion

and mixing of food particles with digestive fluids (McLean 1970), food particles can be retained for relatively long periods of time (Campbell 1965; Day & Cook 1995; Harris et al. 1998a). Food particles arrive in the crop as small fragments that have been grated by the serrated radula that extends from the buccal cavity through the oesophagus (Campbell 1965).

In *Haliotis laevigata*, the crop lumen is an anaerobic to microaerophilic environment (Harris et al. 1998b), and the abalone crop lumen has been shown to be the most acidic region of the digestive tract in both *H. laevigata* (pH 5.3) and *H. midae* (pH 5.6) (Erasmus et al. 1997; Harris et al. 1998b). Due to the relatively long retention time of food in the crop, which is non-ciliated for the most part (Campbell 1965), and the relatively low pH, which could reflect the formation of volatile short-chain fatty acids as fermentation end-products, the abalone is likely to be a foregut fermenter (Day & Cook 1995) rather than a hindgut fermenter like most herbivorous fishes (Mountfort et al. 2002). Acetic acid and formic acid are the main fermentation by-products produced by seaweed-fermenting bacteria isolated from the guts of abalone (Sawabe et al. 2003), but the location of their formation in the abalone gut has not been established. Generally, the fermentation of fibrous diets with a low nutrient density is facilitated by a symbiotic association with gut bacteria (Hungate 1975; Plante et al. 1990). Specialist symbiotic resident gut bacteria in abalone would probably be located at non-ciliated sites of the digestive tract which are both selective and where nutrients are stored for longer periods of time (Harris 1993; Harris et al. 1998a).

In *H. laevigata*, culturable isolates of bacteria decreased in diversity within the most acidic regions of the gut (crop, stomach and style sac), and therefore bacteria in the crop might be limited to fermentative bacteria due to selective pressure (Harris et al. 1998b). Abalone may possess a digestive model that is similar to that of ruminants (Sawabe 2006) if symbiotic resident bacteria ferment food within the selectively acidic abalone crop (foregut). Alternatively, the abalone digestive system could employ characteristics of both foregut and

hindgut fermenters: despite only one storage organ (the crop), abalone also have an extended hindgut with a relatively long and organised intestine (Campbell 1965) which would promote hindgut fermentation (Mountfort et al. 2002). However, the abalone hindgut, which is a ciliated long thin tube, increases in bacterial diversity compared to the crop (Erasmus et al. 1997; Harris et al. 1998b) and therefore seems less likely to be a selective niche chamber for the establishment of a resident gut-bacterial community.

Abalone are able to degrade a wide range of macroalgae and microalgae, as well as formulated pelleted feeds, due to their wide spectrum of endogenous carbohydrases (Bennet et al. 1971; Knauer et al. 1996; Erasmus et al. 1997) and proteases (Picos-García et al. 2000; Garcia-Esquivel & Felbeck 2006). Brown macroalgae has a high content of soluble fibres which include alginate (1,4-linked β-D-mannuronic and α-L-guluronic acids) and fucoidan (1,2-α-L-fucose monomers) as structural carbohydrates, and laminarin (1,3-β-D-glucose monomers) as a storage carbohydrate (Haug et al. 1967; O'Sullivan et al. 2010). Cellulose (crystalline 1,4-β-D-glucans) is the non-soluble fibre in brown macroalgae, and other major components are minerals and water (Mabeau & Fleurence 1993; Lahaye & Kaeffer 1997).

The association between abalone and gut bacteria

Despite possessing endogenous digestive enzymes suited to the macroalgal diet, a symbiotic relationship with fermentative *Vibrio* bacteria has been suggested for abalone, including *H. midae* (Sawabe et al. 2003), fed a natural diet of brown macroalgae or alginate-containing formulated feed (Sawabe et al. 1995; Sawabe et al. 1998; Sawabe et al. 2003; Sawabe 2006). This symbiotic relationship is formed due to the possible inability of abalone to degrade the polygulunorate building blocks of the alginate hetero-polymer in brown macroalgae, which is digested by marine bacteria, whereas the polymannuronate blocks are digested by abalone endogenous enzymes (Boyen et al. 1990; Sawabe 2006; Dong et al. 2012). Furthermore, bacteria isolated from the gut of *H. midae* were able to degrade various polysaccharides in

macroalgae and it was therefore proposed that gut bacteria aid in the digestion of macroalgae (Erasmus et al. 1997). The highest activities for bacterial alginolytic and cellulolytic enzymes were found in the crop (Erasmus et al. 1997), which supports the suggested rumen-like model for abalone digestive systems (Sawabe 2006).

Although evidence exists of a symbiotic relationship between abalone and their gut bacteria, it is not known whether the abalone gut contains mostly transient bacteria or residents that contribute actively to the host metabolism. Scanning electron microscopy analysis of the gut epithelium of greenlip abalone *H. laevigata* established that bacterial-host relationships seemed limited to transient associations due to the failure of bacterial attachment to gut epithelial surfaces, and this was attributed to the large volumes of mucus that was associated with the epithelium (Harris et al. 1998a). The prominent occurrence of mucus, continually secreted by the salivary glands, oesophageal pouches and mucosal cells (Campbell 1965; McLean 1970) would pose a threat to bacterial attachment due to rinsing and the mucus could also incorporate antimicrobial substances (Harris et al. 1998a; Ellis 2001; Bron et al. 2012). Minimal associations between gut bacteria and Australasian abalone species were also attributed to the abalones' natural diet of red macroalgae, which is easily digestible compared to brown macroalgae, and its utilisation would therefore require less of a dependency on exogenous enzymes for efficient digestion (Foale & Day 1992; Day & Cook 1995; Harris et al. 1998a; Hayashi et al. 2003). Fermentative bacteria from the *Vibrio* genus were also isolated from Australasian abalone (*H. laevigata*) but constituted smaller populations of the abalone gut compared to the communities that have been isolated from abalone that feed on brown macroalgae (Hayashi et al. 2003).

In vertebrates, the gastrointestinal microflora account for an important proportion of metabolic activity in the host through symbiotic host-bacteria associations to the extent that the gut microbiota is viewed as an additional organ (O'Hara & Shanahan 2006). Terrestrial

ruminants and termites eating cellulose-rich diets rely on symbiotic resident bacteria for the digestion of cellulose and for synthesizing proteins (Stingl et al. 2005; Russell et al. 2009). The presence of these symbiotic residents also give rise to other bacterial groups that utilise some of the end products released by the primary degraders of cellulose through metabolic cross-feeding (Flint et al. 2008; Flint et al. 2012). Bacterial fermentation of complex polysaccharides such as cellulose produces energy in the form of short-chain fatty acids. The contribution of energy available to the host that is derived from short-chain fatty acids produced by microbes varies with species and can range from 10 % in humans to 70 % in ruminants (Bergman 1990). Associations between microbes and terrestrial hosts exist partly due to the fact that the host gut provides a unique and favourable aquatic environment which is different to that of the external environment, but this is less likely to be the case with aquatic hosts (Harris 1993). Ingestion, which is a transient relationship between gut bacteria and the host, is the most common type of association between aquatic invertebrates and microbes (Harris 1993). Food-associated ingested transient microbes can pre-condition the food to increase its nutritional value, and can form relatively stable gut communities due to repeated survival through gut passage and proliferation in favourable regions (incubation) prior to excretion (Harris 1993).

South African abalone produce endogenous cellulases (Erasmus et al. 1997), and may therefore be able to digest cellulose on their own. Abalone might employ a similar strategy to that of wood-eating catfishes, for which the lack of a symbiosis with cellulolytic bacteria was attributed to their utilisation of more easily digestible soluble polysaccharides (laminarin) as the main energy source (German & Bittong 2009). The dominant structural carbohydrates in brown macroalgae also differ from cellulose in terrestrial plants (Knoshaug et al. 2013) and may have a higher digestibility for abalone (Bansemer et al. 2014). However, there is evidence of bacterial utilisation of at least one of the soluble

polysaccharides in brown macroalgae, namely alginate. A unique relationship between *Vibrio* species and abalone can therefore be found for abalone that feed predominantly on brown macroalgae, and the symbiotic *Vibrio halioticoli* has been isolated from abalone fed brown macroalgae or alginate-containing artificial feed (Sawabe 2006). Advances in microbial technology might also be able to identify other symbionts in abalone as many marine bacteria are currently unculturable and are therefore not yet identified.

Factors that influence host-bacteria relationships in the abalone gut: the effect of formulated feed on abalone

Gut microbial studies in abalone have shown that abalone gut-bacterial communities differ between abalone that are fed macroalgae and those fed artificial feed (Tanaka et al. 2004; Zhao et al. 2012). Artificial feed seems to increase the gut-bacterial diversity in juvenile (one-year old) abalone (Tanaka et al. 2004), while causing a decrease in gut-bacterial diversity in developing (weaning) abalone (Zhao et al. 2012). In addition, for abalone that are fed natural diets, the gut-bacterial community composition corresponds to that of the feed and external environment, whereas the gut bacteria associated with abalone fed an artificial diet form a distinct community from that of bacteria that grow on the feed (Zhao et al. 2012). Analysis of 16S rDNA gene clone libraries of the gut homogenates of *H. discus hannai* fed artificial feed revealed that *Arcobacter* species (class: epsilonproteobacteria) were unique to abalone fed an artificial diet. *Vibrio* species (class: gammaproteobacteria) and Mollicutes, which are also prevalent in abalone fed fresh macroalgae, were well-represented in the guts of artificial-diet fed abalone (Tanaka et al. 2004).

It therefore seems that farmed abalone can maintain a relatively stable core gut microbiome when fed different diets despite increases in opportunistic bacteria when abalone are fed artificial feed. However, increases in bacterial diversity and bacterial proliferation beyond a natural state may disrupt the balance of the microbial community and pose a threat

to the host immune system and physiology, especially when external conditions become unfavourable (Vandepeer 2006). The reason for the abalone gut-bacterial community shifts in response to artificial feed is not known, although it is likely that typical abalone gut-bacterial communities are shaped by the low nutrient-density of their natural macroalgae diet and changes in the gut microbiota therefore occur in response to an increase in nutrient density. In artificial feed, refined starch of terrestrial plant origin, which is composed of amylopectin (1,6-α-glucosidic bonds) and amylose (1,4-α-glucosidic bonds), forms a major part of dietary carbohydrates (Chinachoti 1995). The high content of energy-dense, non-fibre, grain-derived starchy carbohydrates in artificial feed is in contrast to the carbohydrate composition of macroalgae, which is rich in fibrous structural carbohydrates (Lahaye & Kaeffer 1997; Sales & Britz 2003).

In humans, the gut microbiota is influenced by high post-prandial luminal concentrations of energy-dense carbohydrates derived from refined starch which is not part of the evolutionary framework of the human diet (Spreadbury 2012). The gut microbiota in humans differ between individuals in non-industrialised countries that consume unprocessed diets of cellular carbohydrates (such as tubers and potatoes), and that of westernised individuals that consume grain-derived high-density carbohydrates (De Filippo et al. 2010). The latter group was found to have a gut microbiota that interferes with normal metabolism, and this gut microbial association type can be viewed as an “inflammatory gut microbiota”, which deviates from symbiotic co-evolutionary gut microbial associations (Spreadbury 2012). Bacteria that proliferate in response to energy-dense diets and yield subsequent increases in bacterial lipopolysaccharide molecules produce toxic effects in the host which interfere with normal endocrine responses and their role in the overall metabolism (Ghanim et al. 2009; Erridge 2011; Lassenius et al. 2011; Spreadbury 2012). Similar implications could be hypothesised in abalone; with distinct bacterial community and physiological

responses between abalone fed high-energy carbohydrate-dense artificial diets, which may be associated with the bloating found in abalone fed formulated feed during warm temperatures (Vandepeer 2006), and those fed fibre-rich macroalgal diets. A similar phenomenon is observed in ruminants: animals fed an energy and carbohydrate-dense grain diet instead of their natural fibrous plant diet, can develop bacterial overgrowth and a build-up of lactic acid in the rumen resulting from increased bacterial fermentation rates, which leads to acute or chronic acidosis and further establishments of acid-resistant pathogens (Russell & Rychlik 2001).

Compared to artificial feed, brown macroalgae contains low levels of protein (Fleurence 1999), which is a limiting factor for growth in abalone and herbivores in general (Mattson 1980; Britz 1996). Therefore, protein level should to some extent account for higher growth rates reported for some abalone species fed artificial feed compared to those fed single-species macroalgae diets (Viana et al. 1993; Britz 1996). Amino-acid balance, which could account for differences in growth between abalone fed single-species and mixed-species macroalgal diets (Bansemer et al. 2014), may also be responsible for superior growth in some abalone species fed formulated feed compared to those fed single-species macroalgae diets. Abalone are relatively efficient at converting protein from fishmeal, soybean meal and algal sources to growth. Britz (1996) established protein efficiency ratios (PER) of 3.9, 3.4 and 3.0 for fishmeal, soya oil cake and *E. maxima*, respectively. However, there seems to be a discrepancy between the relatively high inclusion levels of protein in formulated abalone feeds and the relatively low endogenous protease activity levels in abalone (Garcia-Esquivel & Felbeck 2006). Apart from an increase in protease activity in the lower regions of the abalone digestive tract when formulated feed-fed farmed abalone are supplied with probiotics (Macey & Coyne 2005), the role of gut bacteria in the digestion of protein in abalone fed artificial feed is not known. Bacterial digestion of protein in commercially farmed ruminants

leads to wastages when optimal production is aimed for through efficient feed utilisation (Chalupa 1975; Brock et al. 1982). Since protein is more difficult to digest compared to carbohydrates, high loads of protein might serve as a nutrient source for gut bacteria even after carbohydrates are digested. Exogenous proteases can be threatening to the host as they have been implicated as virulence factors in aquatic animals and may lead to tissue damage which leads to infection (Secades et al. 1999).

Dietary-induced disruption of the natural abalone-bacterial associations could place an increased energetic demand on the innate immune system. It has been proposed that the absence of a well-developed adaptive or specific immune system in primitive invertebrates leads to a limited scope for co-evolutionary symbiotic relationships between resident bacteria and invertebrates, for which discrimination between potential pathogens and commensals is required (Mcfall-Ngai 2007). To control the invasion and proliferation of opportunistic and potentially pathogenic bacteria, abalone might have to rely on continuous inflammatory responses, which would drain metabolic energy (Dibner & Richards 2005). In addition, increases in bacterial diversity could give rise to the growth of individuals that are able to resist the host's natural defences, through for example, the degradation of tissue-protective mucus layers (Schroers et al. 2009). Artificial feed, by virtue of its nutritional composition, harbours the potential to promote a gut microbiota with an uncontrolled diversity due to the establishment of generalist opportunists which may pose an energetic challenge to the abalone host.

Possible implications of controlling the gut bacteria of farmed abalone

Bacteria associated with vertebrates can influence the expression of genes involved in the immune system through pathogen-associated molecular patterns (PAMPS) (Wang et al. 2011) so that the host becomes conditioned to commensal microbes for quick recognition of invaders (Gómez & Balcázar 2008). Promoting the growth of resident commensal bacteria

can also protect the host from the establishment of pathogenic invaders by competing with them for resources (Gómez & Balcázar 2008). The presence of bacteria in the gut might also have an effect on the expression of genes involved in other physiological roles, especially during the host's developmental phase. It was found that bacteria play a role in the development of gut tissue in zebrafish through upregulation of the genes involved in gut development (Rawls et al. 2004). Similarly alterations in the gut microbiome displayed differential effects on growth in developing larval *Drosophila* (Shin et al. 2011). Disruption of the normal gut microbiome in farmed abalone might therefore also have an effect on the normal development and physiology of abalone.

In addition to diet, gut bacterial communities are influenced by physiological stress and other external conditions. Thus bacterial control through dietary intervention potentially becomes an important strategy to mitigate the adverse effects that imbalanced gut-bacterial communities can have on the physiology of farmed animals. Gut bacteria are able to compete with the host for nutrients such as vitamins (Harris 1993) and can produce both beneficial (such as short-chain fatty acids, vitamins and activation of bioactive compounds) and harmful metabolites (such as ammonia, hydrogen sulphide and phenols) through metabolic transformations of nutrients and chemicals (Blaut & Clavel 2007). The growth of resident bacterial groups in abalone should be regulated as naturally occurring *Vibrio* species can become pathogenic when environmental or physiological conditions change (Liu et al. 2000). Depending on the multiplication rate of the species, optimal micro-environmental conditions for bacterial growth should be avoided to prevent gut-bacterial overgrowth. In addition, gut bacterial growth and increases in bacterial fermentation and gas production in formulated feed-fed abalone in response to increases in water temperature has been implicated as a contributing factor in summer mortality (Vandepeer 2006).

To establish means for regulating gut bacterial growth in abalone, the use of probiotics has been investigated. Probiotics have been defined as live microbial supplements that remain alive through passage through the digestive tract to exert beneficial effects on the host through gut-bacterial modulation, improvement of feed utilisation and through an enhanced response to disease (Gatesoupe 1999; Verschuere et al. 2000). In formulated feed-fed *H. midae* supplied with probiotics (enzyme-secreting bacterial and yeast strains isolated from the abalone gut and cultured in the lab), both growth and survival was increased and protease activity in the intestine was elevated in correlation with a higher degree of protein digestion (Macey & Coyne 2005). Increased survival in abalone supplemented with probiotics is achieved through increases in immune competency through an increased number in circulating leukocytes with higher activity levels when abalone are challenged with a pathogen (Macey & Coyne 2005; Jiang et al. 2013). Probiotic supplementation can effectively increase growth in abalone fed both artificial feed (Macey & Coyne 2005) and macroalgal diets for host-derived cultured *Pseudoalteromonas* species probionts and for enzyme-secreting cultured *Vibrio* and *Agarivorans* probionts isolated from the digestive tract of *H. rufescens* and from macroalgae (ten Doeschate & Coyne 2008; Silva-Aciares et al. 2011). Probionts can increase the digestibility of macroalgae as increased alginate lyase activities were found in the crop and stomach of abalone fed a probiotic-supplemented kelp diet compared to those fed a non-supplemented kelp diet (ten Doeschate & Coyne 2008). *Haliotis gigantea* fed artificial feed supplemented with cultured probiotic strains of lactic acid bacteria were able to produce elevated levels of short-chain fatty acids in the gut and displayed increases in protease activities (Iehata et al. 2009) and alginate lyase activities (Iehata et al. 2010). In addition, supplementation with host-derived lactic acid bacteria in the diets of *H. gigantea* resulted in a change in the culturable (Iehata et al. 2010) and overall gut-bacterial composition (Iehata et al. 2014).

Even though the probiotic bacteria that have been tested for *H. midae* are able to effectively colonize the abalone gut, farmed abalone would need to be supplied with probiotics every second day to sustain effective numbers of probionts in the gut (Macey & Coyne 2006). To produce probiotics for commercial use, the conditions for their growth and production on a large scale would need to be established in addition to methods to ensure their preservation, safety, quality, and efficacy in the farm environment through extensive in vivo experiments to establish their interactions with the host and farm system (Verschuere et al. 2000; Balcazar et al. 2006; Moodley et al. 2014). To date, the use of probiotics have not been implemented for commercial abalone culture due to the cost implication of its practical application on-farm, and therefore a "prebiotic" or seaweed-supplement vector approach to maintain stable gut-bacterial communities in farmed abalone may be more practical.

The effect of a combination diet of formulated feed and macroalgae on cultured abalone

It has been shown that seaweed can replace a portion of fishmeal in the artificial diets of abalone without compromising growth (O'Mahoney et al. 2014), and therefore combination diets of formulated feed and macroalgae can improve the sustainability of abalone feeds by reduced fishmeal usage. In addition, such combination diets can produce growth rates in farmed abalone that are higher compared to that of abalone that are only fed formulated feed (Durazo-Beltrán et al. 2003; Dlaza et al. 2008) (See Bansemer et al. (2014) for a review on the use of seaweed in abalone feed). However, the physiological mechanisms behind these increases in abalone growth remain unknown.

Haliotis rufescens abalone fed a combination of formulated feed and brown macroalgae only out-performed abalone fed formulated feed in terms of growth when a relatively high inclusion level of fresh macroalgae was added to the diet (Kemp et al. 2015). In addition, these authors found that the utilisation of protein in the formulated feed was improved with the addition of macroalgae to the diet. Feed utilisation of artificial feed components in *H.*

midae diets are also improved when mixed seaweeds (kelp, *Ulva* and *Gracilaria*) are used in combination diets with formulated feed (Justin Kemp, unpublished data). Although the physiological mechanisms behind increases in feed utilisation and growth for abalone fed macroalgae-formulated feed combinations remains unknown, Kemp et al. (2015) suggested that the cause could be a combination of macroalgal derived micro-nutrients, bioactive compounds and gut-bacterial regulation associated with exogenous enzyme production.

Providing farmed abalone with combination diets of formulated feed and macroalgae might serve as the optimal strategy to improve abalone growth, feed utilisation and health. Optimising dietary supplementation of seaweed for abalone fed formulated feed is important for the culture of locally farmed *H. midae* and for Australian abalone species which are mainly produced using formulated feeds. In contrast, seaweed is the main food source for sub-adult and adult abalone cultured in China and those cultured in Chilé (Zhao et al. 2012; Kemp et al. 2015). While formulated feed may promote abalone growth through its balanced macronutrient composition and nutrient-density, a macroalgae supplement might improve feed utilisation by regulating the abalone gut microbiome and its associated enzymes.

The use of prebiotics has not been tested for farmed abalone, but macroalgae might contain compounds with prebiotic properties in abalone. A candidate prebiotic would have to be a compound that is not digested by abalone endogenous enzymes and is instead selectively fermented by resident gut microbes (Gibson et al. 2004), for example, guluronic acid in alginate. Brown macroalgae, for which fragments can be retained in the crop for up to 48 hours (Day & Cook 1995), long after the ingredients of formulated feed have been digested, could sustain the establishment of a healthy resident gut microbiome. This would add to the other health benefits that dietary macroalgae introduce in abalone, such as its contribution to the antioxidant status in farmed abalone (Bansemer et al. 2014; Stone et al. 2014) to promote overall health and performance.

Seaweed supplementation has also been shown to be beneficial for a range of other unrelated animal species. In both the pig and fish farming industries, seaweed and its extracts are included in formulated diets to increase feed utilisation through modulation of gut-microbial communities and to delay the absorption of nutrients (Yone et al. 1986; Hoebler et al. 2000; O'Doherty et al. 2010; Stadtlander et al. 2013). Soluble fibres from brown macroalgae act as prebiotics in both humans and piglets, and subsequently improve growth and feed conversion in piglets (Terada et al. 1995; O'Doherty et al. 2010). In fish, a favourable composition of gut bacteria improves growth (Gatesoupe 2008; Bagheri et al. 2008; Marzouk et al. 2008; Maity et al. 2011). Similarly, in farmed shrimp and sea bass, increased growth through prebiotic supplementation (0.1 % and 4 % inclusion) corresponded with changes in the gut microbiota (Zhou et al. 2007; Torrecillas et al. 2007).

The kelp, *Ecklonia maxima*, is currently included at relatively low levels in a trademarked locally-developed formulated feed which is supplied to farmed *H. midae*. However, the optimal level of dietary kelp inclusion in the formulated feeds of farmed *H.*

midae has not been established, and relatively low kelp inclusion levels are used in industry to maintain the integrity of formulated feed pellets.

Other health benefits of brown macroalgae in abalone feed

As a supplement in formulated abalone feed or included in its fresh form as a component in combination diets, brown macroalgae is likely to have beneficial physiological effects beyond its possible role as a modulator of gut microbes. A range of soluble fibres and bioactive compounds are unique to brown macroalgae. The polyelectrolyte soluble fibres it contains may promote the bioavailability of minerals (Lahaye & Kaeffer 1997) as soluble fibres (inulin) in dairy infant formulas promote calcium availability (Bosscher et al. 2003), and similar mechanisms in abalone could prevent macro-mineral deficiencies in cultured abalone. In vertebrates, these soluble fibres also interact with growth-factor components of the epithelial layer of the gastrointestinal tract through molecular positioning, which leads to cell proliferation and increased absorptive surface areas in the gut (Lahaye & Kaeffer 1997).

Fucoidans have a strong biological activity which include antiviral, anti-inflammatory and antioxidant activities (Li et al. 2008). Seaweed compounds can have direct and indirect effects on bacterial growth inhibition. Some β-glucans, such as those found in laminarin act as immunomodulators and are referred to as immunosaccharides: β-glucans interact with receptors on the surface of innate immune cells like macrophages which signal bactericidal activities performed by phagocytes, lysozymes and reactive oxygen species (Sweeney et al. 2012; Song et al. 2014). Other seaweed components, such as terpenoids, alkaloids and phenolics are produced as secondary metabolites as a chemical defence against herbivory and parasitism by microbes and are selectively bacteriostatic and bactericidal (Steinberg et al. 1997). This could have implications for the regulation of bacterial growth in abalone when seaweed, which then acts as a natural antibiotic, and bacteria are both present in the abalone gut (Dierick et al. 2010). Compared to other kelps in South Africa, *E. maxima* has a

particularly high content of polyphenols in its meristoderm which can reach levels of 39 % on a dry mass basis (Tugwell & Branch 1989).

At first glance, the most prominent difference between abalone fed artificial feed and those fed macroalgae is the lack of colouration in the cultured abalone shell due to the lack of macroalgal pigments in their diet. Clearly, the limited species composition of microalgae growing in the baskets of locally cultured abalone is not contributing to shell colouration in abalone. Since pigments are retained in the gonads they may be important in reproductive biology (Maoka 2011). Pigments can play a large role in metabolism as prosthetic groups of vitamins and other co-factors (Delgado-Vargas et al. 2000). Carotenoids such as fucoxanthin in brown macroalgae are reactive chained molecules that react with singlet oxygen and free radicals and are efficient antioxidants that prevent cell and tissue damage in animals (Miyashita et al. 2011). Phenolic compounds in brown macroalgae also display antioxidant activities (Lim et al. 2002; Jiménez-Escrig et al. 2012). Antioxidants can play a very important role in reducing the effects of oxidative stress in farmed abalone in response to environmental stressors on the farm (Lange et al. 2014).

Farmed abalone that are fed formulated feeds supplemented with macroalgae would also feed on microalgae that grow naturally in abalone culture systems, but macroalgae seems to provide an additional nutritional or pharmaceutical benefit. In contrast to macroalgae, microalgae are composed of simple or short-chain carbohydrates that would be easily degradable by digestive enzymes compared to macroalgae (Markou et al. 2012) and are therefore less likely to contain prebiotic compounds. It would seem that incorporating the abalone's natural macroalgae diet in abalone production systems offers a number of potential advantages for abalone health.

Summary

Since abalone can degrade macroalgae through enzymes that are from both endogenous and exogenous sources, it seems that the presence of alginate in abalone diets may provide an opportunity for the establishment or maintenance of symbiotic gut bacteria and subsequent competitive exclusion of pathogenic invaders. Dietary bacterial modulation becomes important in abalone fed energy-dense and nutrient-rich diets. However, the modulatory effectiveness of different dosages of macroalgae supplementation, which would provide a selective fibrous bacterial substrate or act as a natural antibiotic in abalone fed nutrient-rich artificial diets (that promote the growth of opportunistic bacteria), needs to be established. Currently, there exists a vast knowledge gap regarding the physiological mechanisms behind increases in growth and feeding efficiencies in abalone supplemented with macroalgae, especially since these effects can be found for abalone supplemented with different types of macroalgae (brown, red and green) which have distinct biochemical compositions. The role of macroalgae as a contributor of antioxidants in abalone fed formulated feeds is promising, but macroalgae supplementation is likely to exert a cumulative combination of beneficial physiological effects in cultured abalone fed artificial diets.

RESEARCH AIMS AND OBJECTIVES

The aim of this study was to investigate the effects of kelp supplementation on farmed South African abalone fed an artificial diet with respect to growth, gut-bacterial communities, digestive enzyme activities and morphological parameters of the crop.

The objectives were:

1. To test the commercial value of supplementing the formulated feeds fed to cultured sub-adult abalone with relatively low levels of kelp while establishing the optimal inclusion level of dietary kelp supplementation (0.44 – 3.54 % dry mass) (Chapter 2).

2. To compare the crop epithelial thickness between farmed abalone fed a commercial kelp-supplemented formulated feed (0.88 % kelp on a dry mass basis; "KS diet") to those fed a control formulated feed (0 % kelp on a dry mass basis; "control diet") (Chapter 3) in order to establish possible differences in the integrity and cell growth of crop tissue.

3. To compare the levels of digestive enzyme activities between farmed abalone fed the kelp-supplemented formulated feed ("KS diet") to those fed the control formulated feed ("control diet") (Chapter 4). This comparison was performed to establish if kelp supplementation leads to different enzyme activity levels in abalone compared to that of abalone fed the control diet, which could potentially relate to differential communities of enzyme-secreting bacteria in the gut. A supplementary proteomic analysis of digestive system samples of abalone fed the KS diet was performed to identify enzymes of abalone endogenous and exogenous bacterial origin.

4. To compare the gut-bacterial communities between farmed abalone fed the "KS diet" to those fed a "control diet" through molecular metagenomic analyses (Chapter 5), in order to establish if kelp supplementation leads to gut-bacterial modulation in farmed abalone.

In the literature, there is some ambiguity regarding the term "gut" which could either refer to the whole digestive tract or the intestine. In this thesis, the term "gut" refers to the whole digestive tract which includes the oesophagus, crop, stomach, style sac and intestines.

CHAPTER 2

THE EFFECT OF GRADED LEVELS OF KELP, AS A DIETARY SUPPLEMENT, ON THE PRODUCTION OF CULTURED SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*

INTRODUCTION

Seaweed in either a fresh, powdered or dried form has been used as a supplement to formulated feeds fed to farmed abalone. It has been suggested that macroalgae can improve the feeding activity, feed utilisation, growth, health and marketability of farmed abalone (Bansemer et al. 2014).

The health benefits of macroalgae include its effect on abalone antioxidant status and antiviral activity and improved survival for farmed abalone exposed to water temperature increases (Dang et al. 2011; Lange et al. 2014; Stone et al. 2014). The presence of macroalgal fragments in addition to formulated feed in abalone culture baskets can stimulate increases in abalone feeding activity (Allen et al. 2006), but this depends on the macroalgae type and levels of phenolic compounds (Winter & Estes 1992; Fleming 1995; Allen et al. 2006).

The growth benefits of feeding macroalgae and macroalgae-formulated feed combination diets are species dependent as some abalone species such as *Haliotis rufescens* grow better on macroalgae (Kemp et al. 2015), whereas others, such as *Haliotis midae* and *Haliotis laevigata* grow better on formulated feed (Britz 1996; Dlaza et al. 2008; Dang et al. 2011). It has been observed that *H. midae* fed combination diets of formulated feed and macroalgae can out-perform abalone fed macroalgae or formulated feed alone (Dlaza et al.

2008), whereas *H. rufescens* fed combination diets of formulated feed and macroalgae do not display improved growth rates compared to macroalgae-fed abalone (Kemp et al. 2015). Diet history and its influence on gut development and associated bacterial flora may influence the ability of abalone to utilise formulated feed as *H. rufescens* used in the study by Kemp et al. (2015) were weaned onto macroalgae instead of formulated feed. A study on juvenile *H. diversicolor* abalone weaned from a diatom diet onto an artificial feed observed that gut-bacterial communities can be disrupted with switches in diet and that bacteria play an important role in feed utilisation during the abalone's developmental phase (Zhao et al. 2012).

Seaweed inclusion in farmed abalone diets can increase feed utilisation and the nutrient bioavailability of the diet. A study on juvenile red abalone *Haliotis rufescens* (~1.1 g body weight) found that utilisation of the protein component of formulated feed increased with increasing levels of fresh seaweed in the diet (Kemp et al. 2015). It was hypothesised that increased bioavailability of dietary protein can be facilitated by exogenous proteases produced by bacteria associated with the fresh seaweed ingredient (Kemp et al. 2015), since host-derived probiotic strains added to abalone diets can increase protease activity levels (Macey & Coyne 2005; Iehata et al. 2009). Another study on *H. discus hannai* found that when formulated feed was supplemented with macroalgae meal, feed efficiency ratios were increased compared to abalone fed macroalgae or non-supplemented formulated feed, despite similar growth rates for abalone between diets (O'Mahoney et al. 2014).

The application of a feeding regime that includes the use of fresh macroalgae on-farm poses biosecurity and logistical challenges (Bansemer et al. 2014). Therefore, in South Africa, the kelp, *Ecklonia maxima*, which is a major component of the natural diet of wild stocks of *H. midae* in the Western Cape (Barkai & Griffiths 1986), is incorporated into the pellets of a commercial formulated feed (Abfeed®-K26) at relatively low inclusion levels.

Abfeed®-K26 was the most widely used commercial abalone feed in South Africa, and proprietary farm trials indicate that it produces better growth rates and feed utilisation efficiency than the equivalent formulated feed with no kelp (Jones et al., unpublished data). However, no reports have been published documenting the performance of South African abalone fed formulated diets with kelp as an ingredient, and the optimal level of dietary kelp supplementation for farmed South African abalone has not been established. Therefore, a long-term growth trial under farm conditions was conducted to test the effect of kelp level in formulated feed on abalone growth.

MATERIALS AND METHODS

Experimental site and system

The study was conducted on a commercial abalone farm in Hermanus on the south west coast of South Africa (34°43´S; 19°22´E) over eight months from July 2013 – March 2014. A pump-a-shore system on the farm supplied seawater to a header tank from which seawater was gravity-fed to abalone tanks (Naylor et al. 2011). The rearing tanks (4.2 x 0.75 x 0.7 m; length x breadth x height) were made of plastic canvas supported by wooden frames and contained between seven or eight abalone baskets in series. All the tanks were supplied with air through a 20 mm polyvinyl chloride pipe punctured at equal distances and secured at the bottom of the tank. Depending on the number of abalone baskets, each tank contained either one or two inflows and one standing pipe for an outflow. Inflow flow-rates were maintained at 400 – 600 mL/s according to farming practices. Abalone were stocked in oyster mesh baskets (70 x 51 x 60 cm; length x breadth x height) which contained seven vertically-positioned square high-density polyethylene plates to add surface area within the basket, and each basket was covered with a horizontally-placed rectangular corrugated fibre-crete feeder plate (60 x 45 cm; length x breadth) placed on top of the vertical plates about 10 cm below

the water surface (Figure 2.1). In line with normal farm practise, abalone baskets were moved to new tanks every two weeks when the tanks were cleaned, and this reduced tank effects.

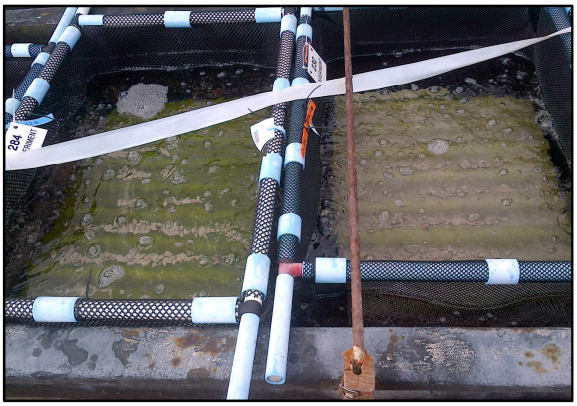

Figure 2.1: The oyster-mesh abalone culture baskets with horizontally placed feeder plates.

Experimental abalone and stocking

Commercially reared sub-adult abalone (*H. midae*) of the same cohort (~43 mm shell length) that had been fed the formulated feed (Abfeed®-S34; 34 % protein, 4.2 % lipid , Marifeed Pty Ltd., Hermanus, South Africa), during their weaning phase (6 - 8 mm shell length) from diatoms, followed by Abfeed®-K26 (~26 % protein, 3.4 % lipid, and a proprietary level of kelp, Marifeed Pty Ltd., Hermanus, South Africa) for approximately five months after being placed in the grow-out section of the farm, were used for this study. During the study, the experimental animals were subject to the farm's standard husbandry practices. At the start of the study in Jul 2013, 30 baskets were stocked with 350 – 400 abalone (g/ abalone with one standard deviation: 19.4 ± 4.5 g S.D) to achieve the target commercial production basket mass for their size range (7070.3 ± 189.0 g/ basket S.D.). The experimental baskets were allocated to different production tanks on the farm. After four months in Nov 2013, the

stocking densities were reduced in line with commercial practise (termed “splitting”) by randomly removing abalone to achieve the required basket masses with 250 – 350 abalone (30.1 ± 7.2 g/ abalone S.D.) per basket.

Diets and experimental design

Five isoenergentic and isonitrogenous experimental diets containing 26 % protein were formulated to contain graded levels of kelp ranging between 0.00 – 3.54 % of dry mass (Table 2.1). These levels were chosen to span a wide range of kelp inclusion levels and to have treatments that are sufficiently distinct from each other, with each inclusion level comprising double the amount of kelp compared to that of the previous level. Kelp was incorporated into the feed by mincing the fresh wet kelp into small fragments (approximately 1 x 4 mm), which were then mixed with the dry ingredients prior to extrusion. All diets were manufactured to the experimental specifications, under the researcher’s supervision, at the Marifeed (Pty) Ltd. feed factory in Hermanus. Each diet was fed to six replicate baskets of abalone using a randomised block design which ensured that each diet was represented only once in each production tank. During the growth trial, feed was supplied once daily before nightfall between 15:00 – 17:00 at approximately 0.3 – 0.4 % of body weight per day according to standard farm practise. The amount of feed applied per basket was recorded daily.

Average percentage leaching (feed applied – feed remaining)/ feed applied x 100 for each diet (N = 2) was established over 48 hours as part of a preliminary experiment for which a system with solid bottom surfaces and manually adjusted airflow and water inflow rates were used.

Table 2.1: The isonitrogenous and isoenergetic experimental diets are displayed for diets with the same protein level (26 % protein) and graded levels of kelp.

	Control Diet	Diet 1	Diet 2	Diet 3	Diet 4
Protein (% dry mass)	**26.00**	**26.00**	**26.00**	**26.00**	**26.00**
Kelp (% dry mass)	**0.00**	**0.44**	**0.88**	**1.77**	**3.54**
Energy MJ/kg	13.80	13.80	13.80	13.80	13.80
Protein: Energy (g protein MJ^{-1})	16.00	16.00	16.00	16.00	16.00
Ingredients (%)[1]					
Fishmeal	17.13	17.12	17.11	17.10	17.07
Soya meal	22.14	22.13	22.12	22.09	22.05
Starch	60.54	59.99	59.45	58.35	56.16
Kelp (% dry mass)	–	0.44	0.88	1.77	3.54
Fish oil %	0.06	0.19	0.31	0.56	1.06
Vitamin mix	0.13	0.13	0.13	0.13	0.13

[1]Percentages for ingredients (including % kelp) are based on the total dry mass of all ingredients, and the following assumptions (based on routine proximal analyses in the past) for each ingredient was used to calculate dietary percentage protein and lipid and energy content respectively: fishmeal 67.5 %, 7.6 %, 193.3 Joule/kg; Soymeal 47.4 %, 2.4 %, 146.4 J/kg; Starch 1 6.4 %, 2.9 %, 160.8 J/kg; Starch 2 7 %, 1 %, 156.9 J/kg; Fish oil 0 %, 100 %, 338.9 J/kg; kelp 10.3 %, 0.7 %, 110.98 J/kg.

Growth measurements

The experimental abalone were reared for a total of eight months, and weighed three times: in Jul 2013 at the start of the experiment, after four months in Nov 2013 and after eight months in Mar 2014. Total weight gain per abalone basket was determined by weighing abalone for each basket in batches on an electronic balance after draining the abalone batches of excess water for 15 min. Sub-samples of thirty randomly selected abalone per basket were individually weighed (0.01 g, Kern PLS 4200-2F, Balingen, Germany) and measured (vernier calipers, accurate to the nearest mm) at the beginning of the experiment and again after four and eight months to establish individual abalone size and variation. Condition Factors (CF) for individually measured abalone were determined using the equation: $CF = 55575 \times (\text{weight} \times \text{length}^{-2.99})$ (Britz 1996). Apparent feed conversion ratio (FCR) was calculated as dry weight feed applied /biomass gain per abalone basket (Naylor et al. 2011), which was not corrected for leaching and uneaten feed loss as the commercial abalone basket design and management practise did not make it possible to collect uneaten food. The amount of uneaten feed was minimised by supplying according to demand, based on feed remaining from the previous night. Similarly, apparent protein efficiency ratio (PER) was calculated as biomass gain/ grams feed protein applied, not corrected for feed waste and leaching losses.

Environmental variables

Sea temperature was recorded 4 – 5 times a week around 08:00 in the morning by farm personnel. The water pH and dissolved oxygen concentrations in the tanks were recorded every 10 to 13 days between 09:00 and 10:30 using an electronic water quality meter (YSI® Pro Plus Multi-parameter meter, Yellow Springs, Ohio, USA). Ammonia was not recorded due to maintenance of tank inflow flow-rates within a commercially optimal range.

Statistical analyses

Normality tests and homogeneity of variances tests were performed using the Shapiro-Wilk test and Levene's test, respectively, and all data analyses were performed using Statistica 12 (Statsoft, Tulsa, Oklahoma). A one-way analysis of variance (ANOVA) was used to establish the effect of kelp level (0 – 3.54 % kelp) on abalone basket mass gain (%). *Post hoc* tests for pairwise comparisons between weighted means were performed using Tukey's HSD test ($p < 0.05$). Non-parametric Kruskal-Wallis ANOVAs ($p < 0.05$) were used to compare individual wet mass (g), individual length (mm), CF, FCR and PER variables between diets due to their failure of the assumptions of an ANOVA test. To establish the relationship between kelp level and basket percentage mass gain, FCR and PER values, linear correlation analyses were performed in Statistica, while curve fittings for best-fit polynomial curves, using the extra sum-of-squares F-test, for FCR and PER in response to kelp level were performed in GraphPad Prism 6.00 (GraphPad Software, San Diego California, USA).

RESULTS

Growth response to graded levels of kelp supplementation in formulated feed

Kelp level had a significant effect on abalone basket mass gain (%) (One-way ANOVA: $F_{4, 25} = 11.42$, $p = 0.000021$) after eight months of growth, for which all kelp-supplemented diets produced significantly higher abalone basket mass gains (%) than the control diet (Tukey HSD; $p < 0.0052$; Figure 2.2). There was a significant trend of increasing mass gain (%) with increasing kelp level ($y = 4199.0 + 332.7x$, $r = 0.66$, $p = 0.000075$, $N = 6$), although there were no significant differences in pairwise comparisons for abalone basket mass gains (%) within the kelp-containing diets (0.44 – 3.54 % kelp) (Tukey HSD; $p = 0.1 – 0.999$).

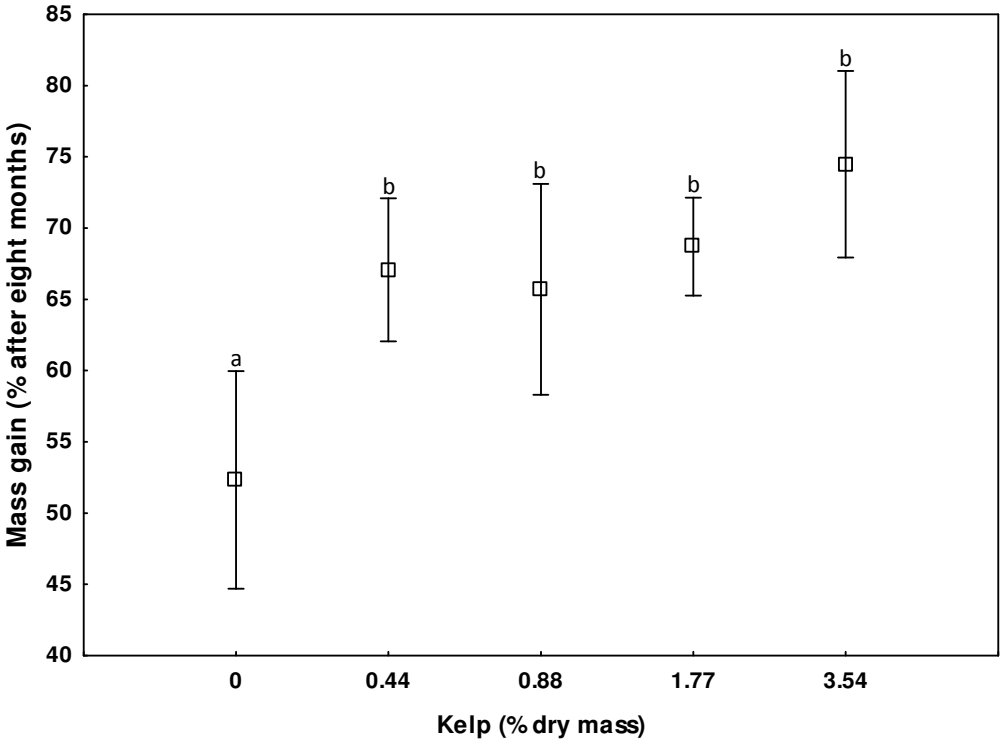

Figure 2.2: Mean abalone basket percentage mass gains with ± 1 S.D. for different kelp levels (N = 6). Different alphabetical letters denote significant differences between kelp levels ($p < 0.05$).

Abalone fed the 0.88 % kelp diet yielded significantly higher individual masses in Mar 2014 than those fed the control diet ($H_{4, 900} = 14.4$, $p = 0.0061$; $z = 3.4$, $p = 0.0069$; Figure 2.3), and no differences were detected in abalone individual masses for pairwise comparisons between the other kelp-containing diets and the control diet or within the kelp-containing diets ($z = 0.74 – 2.5$; $p = 0.09 – 1$).

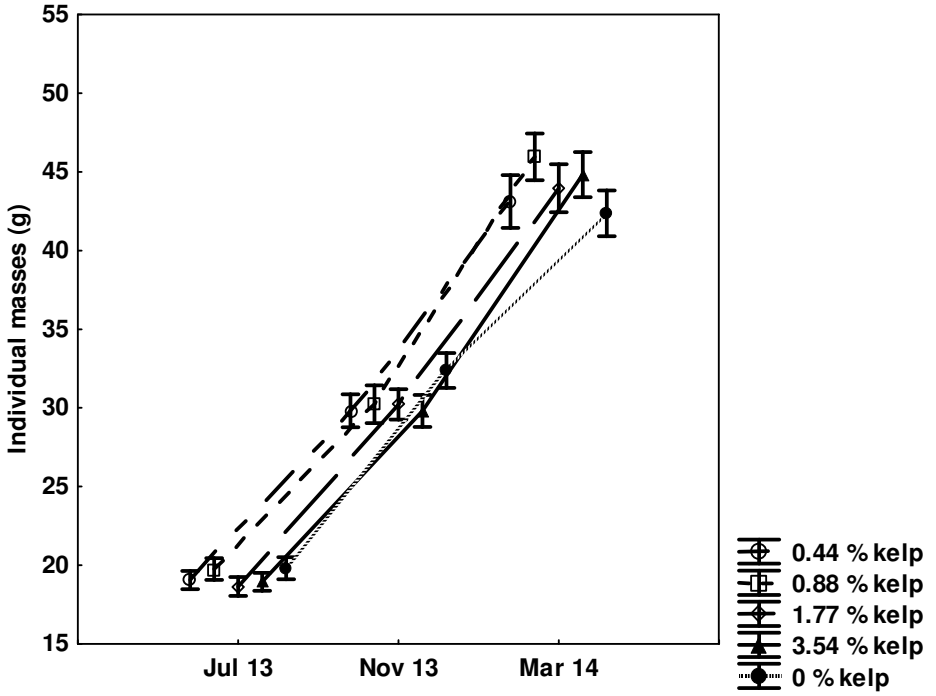

Figure 2.3: Mean individual abalone masses (± 95 % confidence intervals) for basket replicates for abalone fed different kelp levels (N = 180) are displayed. The only significant difference observed was in Mar 2014 (after eight months of growth) between the control diet and the 0.88 % kelp diet and is indicated by the non-overlapping confidence intervals.

No differences in abalone shell length were evident on Mar 2014 between abalone fed the control diet (median with quartile range: 56.1 mm, 52.5 – 60 mm shell length) and the 0.44 % kelp diet (56.0 mm, 52 – 60 mm), the 0.88 % kelp diet (57 mm, 55 – 60 mm), the 1.77 % kelp diet (57.0 mm, 53.5 – 60 mm) or the 3.54 % kelp diet (57 mm, 54 – 60 mm) or between any other pairwise comparisons ($H_{4, 900} = 10.1$, $p = 0.039$; $z = 0.098 – 2.6$, $p = 0.1 – 1.0$) (N = 180). No differences in abalone CFs were found between any of the diets ($H_{4, 900} = 1.9$, $p = 0.75$; $z = 0.015 – 1.3$, $p = 1.0$; Table 2.2).

Kelp level had a significant effect on FCR (Kruskal-Wallis ANOVA: $H_{4, 30} = 21.22$, $p = 0.0003$) and PER (Kruskal-Wallis ANOVA: $H_{4, 30} = 21.21$, $p = 0.0003$) after eight months of

growth, for which abalone fed the control diet displayed a higher FCR than abalone fed the 3.54 % kelp diet ($z = 4.49$, $p = 0.00007$; Table 2.3) and abalone fed the 3.54 % kelp diet displayed a lower FCR than those fed the 0.88 % kelp diet ($z = 2.85$, $p = 0.043$; Table 2.3). Similarly, abalone fed the control diet displayed a lower PER than abalone fed the 3.54 % kelp diet ($z = 4.49$, $p = 0.00007$; Table 2.3) and abalone fed the 3.54 % kelp diet displayed a higher PER than those fed the 0.88 % kelp diet ($z = 2.85$, $p = 0.043$; Table 2.3). Both FCR and PER were significantly correlated with kelp level ($r = -0.70$, $p = 0.00001$, $N = 6$; $r = 0.77$, $p < 0.00001$, $N = 6$, for FCR and PER, respectively), with FCR decreasing (Figure 2.4) and PER increasing (Figure 2.5) with kelp level. FCR and PER values were more variable for abalone fed the control diet compared to those fed the kelp-supplemented diets.

Percentage leaching increased with increasing kelp level ($y = 0.033x + 0.16$, $r^2 = 0.62$, $p = 0.007$, $N = 2$), with a rate of 15.60 ± 7.95 % S.D. for the control feed, and ranging from 16.5 ± 1.21 % for the 0.44 % kelp diet to 27 ± 0.79 % for the 3.54 % kelp diet. The 0.88 % and 1.76 % kelp-supplemented diets had leaching rates of 20.88 ± 3.69 % and 24.38 ± 2.05 %, respectively.

b

Table 2.2: Condition factors (medians with quartile ranges) of abalone fed experimental diets containing graded levels of kelp (N = 180).

Kelp (% dry mass)	0	0.44	0.88	1.77	3.54
Jul 13	1.42 ± 0.018	1.45 ± 0.018	1.40 ± 0.015	1.44 ± 0.022	1.40 ± 0.017
Mar 14	1.38 ± 0.015	1.40 ± 0.014	1.42 ± 0.016	1.41 ± 0.015	1.39 ± 0.013

Table 2.3: Medians with quartile ranges are displayed for apparent feed conversion and protein efficiency ratios (FCR and PER, respectively) for abalone fed diets containing graded levels of kelp. Superscripts denote significant differences between kelp levels (Kruskal-Wallis ANOVA; $p < 0.05$, $N = 6$).

Kelp (% dry mass)	0	0.44	0.88	1.77	3.54
Apparent FCR	2.1 ± 0.36^{a}	1.7 ± 0.12^{abc}	1.8 ± 0.32^{ab}	1.6 ± 0.12^{abc}	1.4 ± 0.12^{c}
Apparent PER	1.9 ± 0.35^{a}	2.3 ± 0.17^{abc}	2.2 ± 0.42^{ab}	2.4 ± 0.18^{abc}	2.7 ± 0.22^{c}

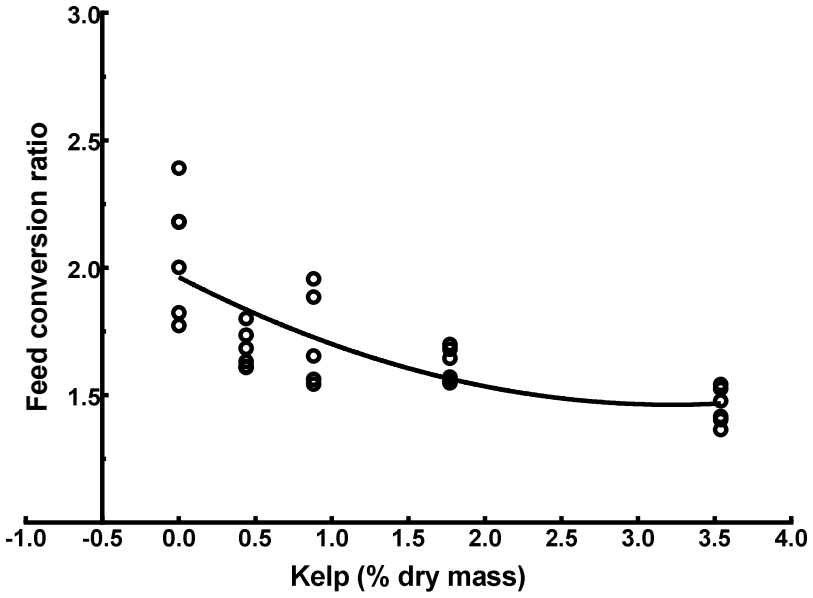

Figure 2.4: A 2nd order polynomial curve for feed conversion ratio (FCR) against kelp level displays a decreasing trend in FCR values with increasing kelp level ($p < 0.001$, $N = 6$).

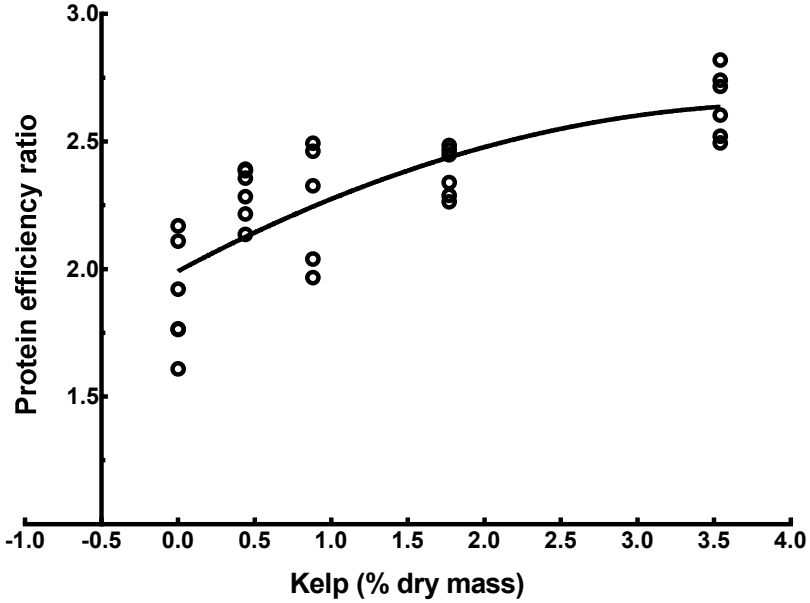

Figure 2.5: A 2nd order polynomial curve for protein efficiency ratio (PER) against kelp level displays an increasing trend in PER values with increasing kelp level ($p < 0.001$, $N = 6$).

Mortality

For the two grow-out periods (Jul – Nov 2013 and Nov 2013 – Mar 2014), mortalities ranged from 0 – 4 and 0 – 5 individuals per abalone basket respectively (< 2 % mortality) and mortality was the same for all treatments.

Environmental variables

Sea temperature averaged 15 °C (14.2 – 16.2 °C, 20.6 °C) (median with quartile range and maximum in parenthesis; N = 145). Medians and quartile ranges for dissolved oxygen (%) and pH in the abalone tanks were 92.5 % (89 – 94.7 %) and 7.9 (7.86 – 8.01), respectively (for inflow and outflow measurements).

DISCUSSION

The presence of kelp, even at low inclusion levels, had a significant positive effect on abalone basket percentage mass gain, FCR and PER relative to that of abalone fed the control diet. This finding is in agreement with two published studies on fresh seaweed-Abfeed® combination diets for *H. midae* grown on-farm (Dlaza et al. 2008; Naidoo et al. 2006). Similarly, farm-reared *Haliotis fulgens* spat grew faster when fed formulated feed in combination with fresh brown seaweed (*Macrocystis pyrifera*) compared to abalone fed formulated feed only (Durazo-Beltrán et al. 2003). In this study, it is possible that physiological adjustments of abalone to the kelp-supplemented feed used prior to the study, which uses a kelp inclusion level that falls within the range of the supplementation levels used in this study, could have contributed to the higher growth rate of the abalone fed kelp-supplemented diets compared to those fed the non-supplemented control.

Biomass gain and feed utilisation efficiency, as indicated by the FCR and PER values, improved with increasing kelp level. In addition, feed utilisation efficiency values were much more variable for abalone fed the control diet, whereas variation was relatively low for those fed the two highest kelp inclusion levels. This could indicate that the presence of kelp in the guts of abalone fed the KS diet stimulates a constant gut environment which may relate to the gut microbiome (see Chapter 1 and Chapter 5). This study indicates that including kelp in formulated feed at the 3.54 % level may be most beneficial for farmed abalone, however, the increased rate of leaching observed in increasing kelp inclusion is potentially a problem in respect of water quality management. The improved feed utilisation in response to dietary seaweed inclusion is in agreement with studies by Kemp et al. (2015) and O'Mahoney et al. (2014) who found that feed utilisation in farmed abalone fed formulated feed is increased when abalone were supplied with fresh macroalgae and a macroalgae meal supplement, respectively.

The reason for the markedly improved abalone growth performance and feed utilisation efficiency with kelp supplementation in this study is not obvious as the macronutrient composition was similar for the experimental diets, with the only distinguishing factor being a low inclusion level of kelp which is comprised mainly of soluble fibres and minerals. Within the literature on abalone nutrition, seaweed supplementation has been suggested to act as a feeding stimulant (Bansemer et al. 2014). A growing body of research suggests that gut bacteria play an integral role in abalone feed utilisation and that the gut bacterial community profile is influenced by diet Zhao et al. (2012). Improved growth rates in *H. midae* abalone through gut-bacterial modulation have been observed for studies on host-derived probiotics (Macey & Coyne 2005; ten Doeschate & Coyne 2008). In addition, these and other studies (Erasmus et al. 1997; Zhao et al. 2012) have identified the involvement of bacterial enzymes in the digestion of food by abalone. Studies on the influence of seaweed supplementation on the abalone gut-bacterial community are lacking, but it has been found that low-level dietary supplementation of seaweed and its extracts can modulate the gut-bacterial composition in farmed pigs (Dierick et al. 2009; O'Doherty et al. 2010; Smith et al. 2011) through prebiotic and antibacterial effects (O'Sullivan et al. 2010).

Since bacterial attachment to surfaces of the digestive tract are challenged by mucus production and probiotic supplements need to be re-introduced to the digestive tract on a continuous basis (Harris et al. 1998a; Macey & Coyne 2006; Chapter 1), dietary modulation of the abalone gut microbiome through the use of seaweeds or prebiotics may be the most practical and effective. As kelp is part of the natural diet of abalone, it may stimulate the growth of a more natural commensal gut microbiota in farmed abalone. It can thus be hypothesised that abalone grew better with the kelp supplement due to its role in modulating the gut bacteria as a biological control agent or prebiotic. By promoting the stability of the core gut microbiota and therefore the inhibition of opportunistic bacteria in the gut, less strain

on the abalone immune system may have a positive effect on digestion efficiency by maintaining an optimal energetic balance in abalone.

CONCLUSION

Kelp supplementation within the relatively low inclusion range of 0.44 – 3.54 (% dry mass) in the formulated feeds of cultured South African abalone produced growth-promoting effects for which the highest feeding efficiencies were found for abalone fed the highest level of kelp (3.54 % dry mass). While the mechanisms by which kelp supplementation enhances abalone growth have not been established, the literature suggests that kelp supplementation may promote gut bacterial populations which maintain an efficient digestive physiology of abalone. The effect of kelp supplementation on bacterial-derived digestive enzyme production and the composition of gut-bacteria were thus investigated (Chapters 4 and 5).

CHAPTER 3

CROP HISTOLOGY IN CULTURED ABALONE, *HALIOTIS MIDAE,* IN RESPONSE TO A KELP-SUPPLEMENTED FORMULATED FEED, AND MICROSCOPICAL CHARACTERISATION OF THE CROP SURFACE

INTRODUCTION

Locally farmed *Haliotis midae* abalone are fed a formulated feed (Abfeed®-K26) which includes the kelp (*Ecklonia maxima*). While the growth trial described in Chapter 2 indicates that kelp supplementation improves abalone growth, the physiological mechanism remains uncertain.

It has been observed that macroalgal fermentation end-products produced by symbiotic-bacterial fermentation in abalone consists mainly of acetic acid and formic acid (Sawabe et al. 2003). These organic acids can enhance growth in juvenile (~23 mm) *H. midae* (Goosen et al. 2011) when added to abalone feeds as a dietary supplement. The formation of short-chain fatty acids by fermentative symbiotic gut bacteria is one of the benefits of prebiotic dietary compounds (Cummings et al. 2001; O'Sullivan et al. 2010; see Chapter 1) and probiotic supplementation in abalone (Iehata et al. 2010).

Animals display phenotypic flexibility to either decrease the rate of cell proliferation to conserve energy when nutrients are limited, or to increase cell proliferation and cell growth to compensate for changes in nutrient bioavailability (Gao et al. 2008). In addition, the presence of short-chain fatty acids in the gut, such as butyrate, can directly promote gut-epithelial cell growth since short-chain fatty acids can act as trophic factors for cells along the

gut (Sakata 1987; Topping & Clifton 2001). To date, there are no published studies on the effects of prebiotic compounds such as those potentially found in macroalgae on the digestive tract histology of abalone, but such studies have been performed on fish species.

Morphological responses to dietary prebiotic supplementation in fish have been observed within the fish intestine. However, the fish species studied probably differ from abalone in that hind-gut fermentation occurs in the intestine, as opposed to foregut fermentation in the abalone crop. A study that tested the effect of graded levels of prebiotic mannan oligosaccharides (MOS) on gut histology in rainbow trout (*Oncorhynchus mykiss*) found increases in villi length in the small intestine of fish supplemented with MOS (1.5 and 3 parts per million) (Yilmaz et al. 2007). Similarly, a histological study on MOS supplementation in the fishmeal-based formulated feeds of gilthead sea bream (*Sparus aurata*) found that MOS increased intestinal microvilli lengths and densities (Dimitroglou et al. 2010).

The potential histological adjustments that can be expected for different regions of the abalone gut in response to different dietary factors are largely unknown since histological studies on abalone are limited. A study on the effects of temperature and dietary protein level on the gut epithelium in greenlip abalone (*Haliotis laevigata*) found that diet could contribute to reducing absorptive surface areas in the gut, and that abalone may display compensatory morphological adaptations to nutrient bioavailability (Schaefer et al. 2013). Schaefer et al. (2013) observed that an increased dietary protein level led to decreases in crop cell height (size) in sub-adult *H. laevigata*, and suggested higher levels of anti-nutritional factors in solvent-extracted soybean meal associated with an increased protein level as a causative factor. There were no reductions found for intestinal villi height and the overall surface area of the intestinal epithelium (Schaefer et al. 2013).

Morphological responses characterised by reductions in absorptive surface areas in the gastrointestinal tract in response to anti-nutritional factors in soybean meal have been recorded for fish species. In farmed Atlantic salmon and Rainbow trout, dietary solvent-extracted soybean meal causes symptoms of soybean meal-induced enteritis in the intestines, which include widening and shortening of the intestinal villi folds and a widening of the villi's central lamina propria which becomes infiltrated with leukocytes (Baeverfjord & Krogdahl 1996; Krogdahl et al. 2003; Heikkinen et al. 2006; Bakke-McKellep et al. 2007; Merrifield et al. 2009).

Negative histological responses in *H. midae* have been observed in the abalone digestive gland in response to a sub-optimal nutrient status, but bacterial colonization of the epithelial cells of the digestive gland of farmed abalone by Rickettsiales (class: alphaproteobacteria) displayed no histopathological effects in the hepatopancreas of *H. midae* (Horwitz et al. 2016). The lumen size in the tubules of the hepatopancreas of *H. midae* can become enlarged in formulated feed-fed abalone that may not have adjusted to formulated feeds adequately during their weaning phase (Justin Kemp, unpublished data).

Schaefer et al. (2013) observed that *H. laevigata* kept at cooler water temperatures displayed thicker stomach epithelia compared to those kept at warmer temperatures. It was suggested that abalone kept at cool temperatures were compensating for the reduced digestive enzyme activities (Edwards & Condon 2001), coupled with reduced nutrient availability at cool temperatures, by increasing the size of epithelial cells in the stomach, which are mostly secretory cells that can produce more digestive enzymes (Harris et al. 1998a).

Kelp-supplemented and non-supplemented formulated abalone feeds which are similar in macronutrient composition are not expected to have differential effects on the nutrient status of cultured abalone apart from possibly effecting differences in the levels of bacterial-derived fermentation by-products. For the present study, it was hypothesised that potential

selective bacterial fermentation of kelp fragments in the crops of abalone fed kelp-supplemented diets might stimulate morphological changes in response to the additional available nutrients in the form of short-chain fatty acids.

The aim of the present study was to establish whether kelp supplementation stimulates cell growth in the crop. The specific objectives were to: 1) compare the heights of epithelial cells in the crop between abalone fed kelp-supplemented and non-supplemented (control) formulated feeds; and 2) to view the inner surface of the crop using scanning electron microscopy (SEM) to establish the presence of bacteria in the crop and their association with the crop surface.

METHODS

Sample collection and preparation

Two isonitrogenous and isoenergetic experimental diets were formulated with a 26 % protein level for the experiments in this chapter and for those of Chapters 4 and 5. The kelp-supplemented diet is referred to as the "KS diet" in this thesis (Table 3.1), and the "control diet" was formulated to exclude the kelp supplement while balancing the macronutrient composition to be similar to that of the "KS diet" (Table 3.1). For the KS diet used in this chapter and in Chapters 4 and 5, a 0.88 % kelp level was chosen due to the low leaching rate recorded and because there were no significant increases in growth for abalone fed kelp-supplemented diets with increasing kelp level (Chapter 2). New abalone culture baskets (the same basket and tank type described in Chapter 2) with new animals (~55 mm) were stocked within a single tank on a commercial abalone farm in Hermanus (34°43´S; 19°22´E; Chapter 2), with three replicate baskets per diet, arranged within the tank according to a randomized block design. The experimental tank contained abalone that had previously been fed a kelp-

supplemented commercial abalone feed that contained fishmeal and soymeal as the main protein sources for at least twelve months prior to the start of the experiment.

Table 3.1: The isonitrogenous and isoenergetic experimental diets, one with kelp (the KS diet) and one without kelp (the control diet) used for abalone sampled for morphological, enzyme and gut-bacterial analyses.

	KS diet	**Control diet**
Ingredients (% dry mass)		
Kelp	0.88	0.00
Fishmeal	17.68	16.88
Soymeal	23.57	24.84
Starch	57.74	58.15
Lipid	0.00	0.00
Vitamins	0.13	0.13

Abalone samples (5 – 12 abalone per sampling event) for the present morphological analyses, as well as for enzyme (Chapter 4) and bacterial community (Chapter 5) analyses were all collected from this tank from all three dietary replicate baskets per sampling time and euthanized at -20 °C. Abalone for histology were collected on 21 October 2014, approximately seven weeks into a four-month growth trial (11 August 2014 – 10 December 2014) after an initial three-week acclimatisation to the diets, and dissected and fixed immediately after collection and an euthanization period of 40 minutes (see below). A seven-week period was chosen due to logistical constraints on the farm and to ensure that experimental abalone were fed by the same person during the feeding trial. During this period the abalone displayed positive growth with average masses of 29.1 – 31.2 g (~55 mm)

in Aug 2014 and average masses of 38.6 – 47.2 (60 – 64 mm) in Dec 2014. Average sea temperature for the period of 11 August 2014 – 21 October 2014 was moderate (average: 15.3 ± 0.84 °C S.D.).

Six randomly-collected abalone (30.02 ± 8.1 g S.D.) from each diet treatment were prepared for histology. The abalone were dissected cold and aseptically to obtain their whole digestive tracts. Whole gut samples were fixed in 10 % formalin with a seawater buffer. Transverse sections through the whole abalone digestive tract were cut dorsoventrally below the base of the gills (Schaefer et al. 2013; Figure 3.1) and were sent to the Veterinary Histopathology Laboratory at the University of Pretoria where slides were prepared according to the method of (Bancroft 2003). Fixed tissue samples were dehydrated by a graded series of ethanol concentrations and chloroform, after which they were embedded in paraffin wax and sectioned at 4 – 5 μm with a rotary microtome. Sections were mounted onto microscopic slides and dried prior to staining with a Haematoxylin-Eosin solution. Slides were viewed with an Olympus BX40 light microscope under 20 X magnification and photographed using an Olympus DP72 digital camera.

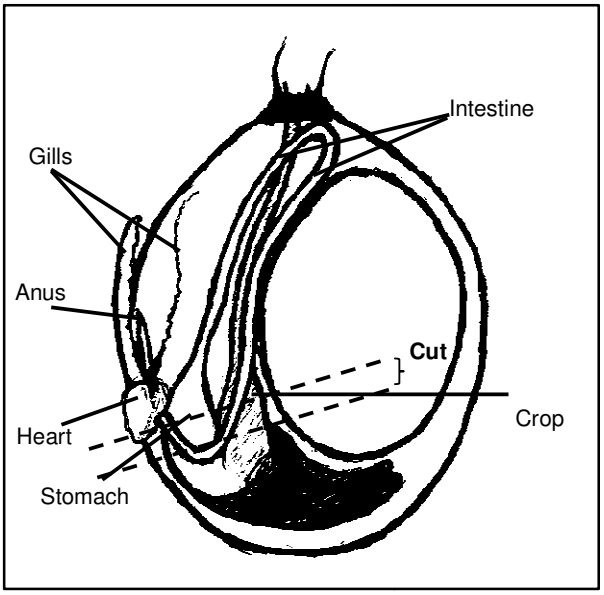

Figure 3.1: The transverse section of the crop was obtained by cutting along the two dashed lines displayed here. The figure is adapted from that of Erasmus et al. (1997).

Abalone (46.2 ± 5.5 g S.D.) that had been fed both diets were collected for SEM samples. For SEM, larger abalone, compared to those collected for histological analyses, were collected from both dietary groups to obtain sufficient organ surface areas for examination. Whole gut samples of five abalone (for which three were from abalone fed the KS diet and the other two were from abalone fed the control diet) were fixed in a 2.5 % gluteraldehyde-filtered seawater solution in which the samples remained incubated for one day at 4 °C. The digestive tracts were then rinsed in seawater twice for ten minutes each time, and whole crop samples were sectioned and spread open and prepared for SEM microscopy using the technique of Cross (2001). Samples were rinsed with 30, 40, 50, 70, 80 and 90 % ethanol for five minutes each to dehydrate the samples, after which they were rinsed in absolute ethanol twice for ten minutes each. The samples were dried with critical

point drying using liquid carbon dioxide, after which they were mounted onto round metal stubs using double-sided tape, and coated with a thin film of gold prior to analysis with SEM.

Histological measurements

Individual crop cell heights were measured according to the method of Schaefer et al. (2013), whereby the distance of the cell from the basal lamina to the gut lumen was measured in ImageJ version 1.46r (National Institutes of Health, USA). The side of the crop abutting the stomach was used for cell measurements (Figure 3.2A). The mean for all cell measurements per crop sample (N = 350 – 1400) was calculated.

Statistical analysis

Statistica 12 was used to calculate normality tests and homogeneity of variances using the Shapiro-Wilk test and Levene's test, respectively, and to compare crop cell height (μm) between abalone fed a kelp-supplemented formulated feed (0.88 % dry mass) and those fed a control formulated feed (0 % kelp) using a T-test.

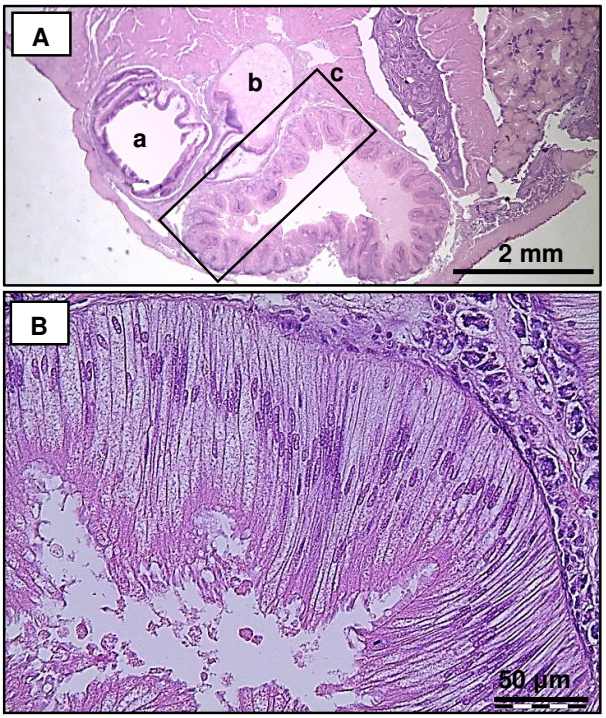

Figure 3.2: A) The rectangular area indicates the side of the crop that was used to measure cell heights (a = stomach, b = intestine and c = crop section); B) The long columnar cells of the crop epithelium are displayed.

RESULTS

Crop cell heights

There were no differences in cell heights between the crop samples of abalone fed the KS diet (Mean ± S.E.: 93.4 ± 10.3 µm; N = 6) and those fed the control diet (109.7 ± 6.6 µm; N = 6) (T-test: t = -1.34, p = 0.21), and no differences were observed for crop fold heights (cell proliferation) or tissue integrity for the different parts of the digestive tract that were visible in the sectioned area.

Scanning electron microscopy

The abalone crop surfaces were lined with mucus, food particles and fragmentation spherules. Filamentous and rod-shaped (~5 μm) bacteria were easily found on the crop surfaces of three abalone for which crops included that of abalone fed both diets (Figure 3.3, Figure 3.4 and Figure 3.5). Bacteria were also observed on the surfaces of the crops of the other two abalone sampled, but Crop 4 (Figure 3.6A) was heavily covered with mucus and only one rod-shaped bacterium was observed, whereas only rod-shaped bacteria were visible on the surface of Crop 5 (Figure 3.6B), which was covered with food slurry. The crop of one abalone fed the control diet (Crop 3) seemed heavily colonized by both rod-shaped and filamentous bacteria or flagellated rod-shaped bacteria (Figure 3.5) compared to the sampled crops of abalone fed the KS diet. Rod-shaped bacteria (~2 μm) found on the feed pellets were shorter than those found within the abalone crop (Figure 3.7). Cilia, which are found in the oesophagus (Figure 3.8A), lower regions of the crop, the stomach and intestines are not to be confused with the filamentous bacteria found in the crop and in the oesophagus of one abalone (Figure 3.8B).

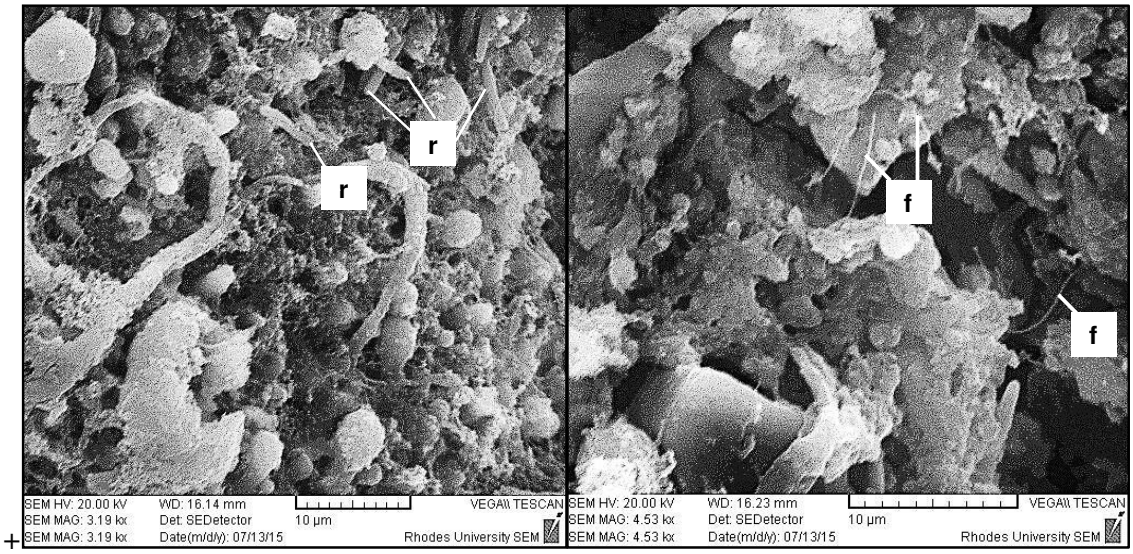

Figure 3.3: Bacteria on the surface of Crop 1 (KS diet) (r = rod-shaped bacterium, f = filamentous bacteria).

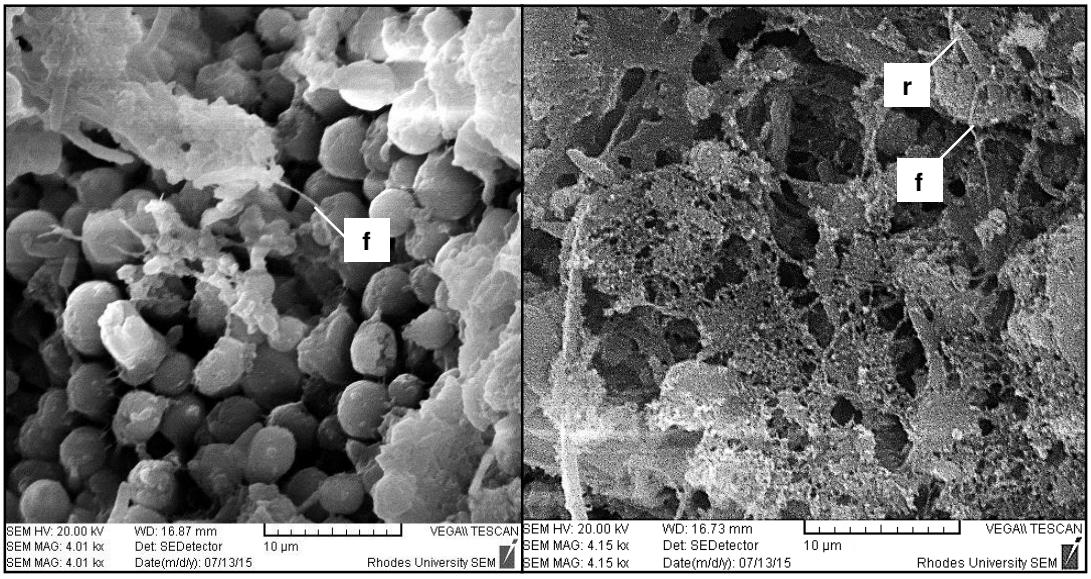

Figure 3.4: Bacteria on the surface of Crop 2 (KS diet) (r = rod-shaped bacterium, f = filamentous bacteria).

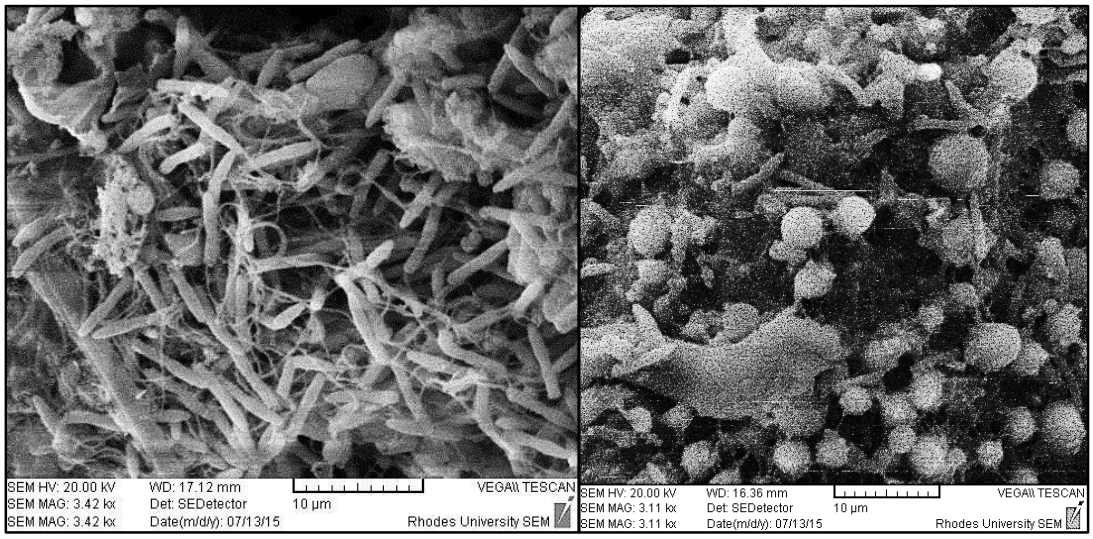

Figure 3.5: Filamentous and rod-shaped bacteria in crevices of the surface of Crop 3 (control diet).

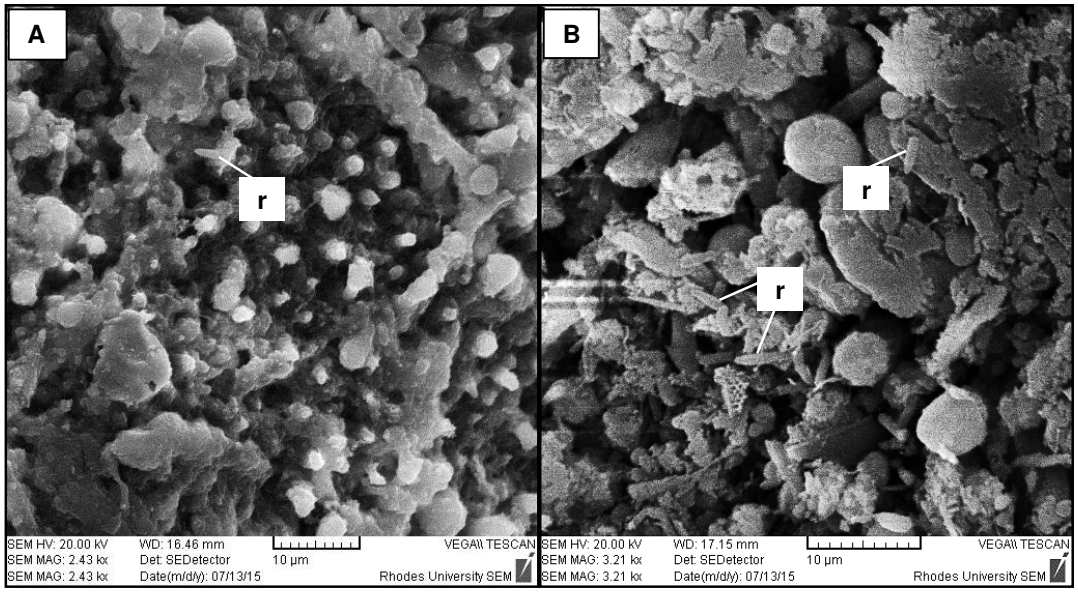

Figure 3.6: Rod-shaped bacteria on the surfaces of: A) Crop 4 (KS diet), and B) Crop 5 (control diet) (r = rod-shaped bacterium).

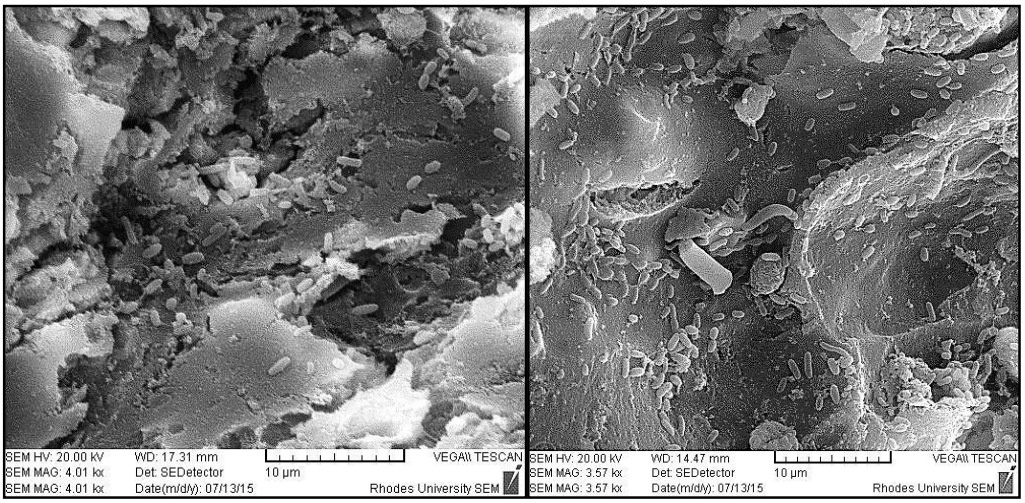

Figure 3.7: Bacteria on the surface of two different abalone feed pellets (KS diet pellet on the left and control feed pellet on the right) that were immersed within the abalone culture tanks overnight.

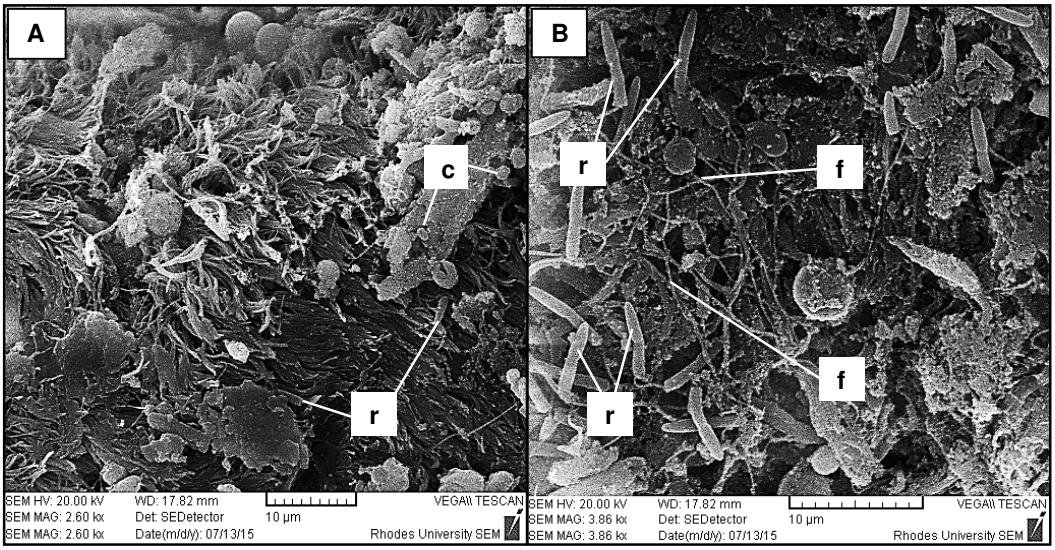

Figure 3.8: A) Rod-shaped bacteria and cocci on the surface of the ciliated oesophagus, and B) larger rod-shaped bacteria and filamentous bacteria associated with mucus in an area of the oesophagus where cilia are not visible (c = cocci, r = rod-shaped bacterium, f = filamentous bacteria).

DISCUSSION

The hypothesis that selective bacterial fermentation of kelp fragments in the crops of abalone fed the KS diet might stimulate morphological changes in response to the additional available nutrients was not supported. The absence of differences in crop cell height, mucosal fold height and observed attached bacteria in the crops of abalone between abalone fed the kelp-supplemented and control formulated feeds may indicate that the two experimental diets were probably too similar in nutrient composition to induce morphological changes. Although there were no gross differences in crop cell height between abalone fed different diets, the abalone sample sizes may have been too small for detecting subtle differences.

The duration of the study over a seven-week period, for which the experimental time period, diet history and similarity in diets formed the constraints of the experiment which were influenced by commercial farming practices, may have been too short for morphological changes to occur. Studies on the effect of prebiotics on morphology have observed increases in the absorptive surface areas of the gastrointestinal tract after only eight and nine weeks for European sea bass and gilthead sea bream, respectively (Dimitroglou et al. 2010; Torrecillas et al. 2011). The only published record of gut morphology in abalone has documented morphological changes after twelve weeks (Schaefer et al. 2013), but no acclimatisation period was reported and there is currently no information on abalone gut morphological changes for studies or sampling events conducted within less than twelve weeks. Alternatively, the crop morphology of abalone fed both diets may have been shaped by their diet history prior to the experimental trial, for which all abalone were fed the "Abfeed®-K26" commercial feed which also included a kelp supplement. Therefore, despite flexibility in morphological adjustments in animals over relatively short periods of time, the core bacterial communities and their fermentation patterns in the crop, that may have been

established in the experimental abalone during earlier life-stages and rearing on previous diets, may have remained relatively stable during the seven-week experiment.

Although it is probable that fermentation of prebiotic substrates, for which kelp is a potential candidate, occurs in the abalone crop, there is currently a knowledge gap regarding possible morphological responses in abalone which makes it difficult to foresee the effect of different dietary factors on abalone morphology. Knowledge on the type of fatty acids produced in the abalone gut in response to different diets is also limited, but it is likely that similar fatty acids may be produced in the gut (Sawabe et al. 2003) despite differential bacterial communities that would be shaped by different diets. Future studies should therefore pair investigations of the effect of prebiotic candidates in the diets of cultured abalone on gut morphology with the levels and type of short-chain fatty acids generated in the gut. In addition, the short-chain fatty acids that affect growth in abalone (Goosen et al. 2011) may be different to those that act as localised trophic factors for cells in the gastrointestinal tract (Topping & Clifton 2001).

The observation of different types of bacteria on the crop surfaces of abalone in the present study differs from observations by Harris et al. (1998a), who found only a single bacterium on the crop surface of one of five sampled seaweed-fed abalone (*H. laevigata*). Filamentous and rod-shaped bacteria seemed to occur commonly within the crop regardless of the diet treatment. Although it is not possible to distinguish differences in the composition of bacterial types with the small sample sizes that were used for this experiment, the rod-shaped bacteria in the crop appeared to differ in size from that of the bacteria that were observed on the feed. This would occur if selective pressure leads to bacterial communities in the crop that differ from that on the feed.

CONCLUSION

The present results were inconclusive, revealing no gut morphological differences between abalone fed kelp supplemented and control diets. This may have been due to the similarity of the diet formulations, the experimental abalones' common diet history and the relatively short experimental period. Both rod-shaped and filamentous bacteria seemed to occur commonly in the crop of *Haliotis midae* fed formulated feed with or without kelp supplementation.

CHAPTER 4

THE EFFECT OF LOW-LEVEL KELP SUPPLEMENTATION ON DIGESTIVE ENZYME ACTIVITY LEVELS IN CULTURED ABALONE FED FORMULATED FEEDS

INTRODUCTION

Abalone, which are marine gastropods, feed predominantly on macroalgae in the wild and have a relatively long digestive tract for the digestion of their fibrous, relatively nutrient-poor natural diet. Ingested and masticated food particles are retained in the crop, where they are stored and mixed with digestive secretions for extracellular digestion (Campbell 1965; McLean 1970; Day & Cook 1995; Harris et al. 1998a) before digested food products are directed to the hepatopancreas and stomach, caecum and intestines (Campbell 1965). The microaerophilic to anaerobic and comparatively acidic (pH 5 – 6) nature of the crop (Erasmus et al. 1997; Harris et al. 1998b) is thought to reflect anaerobic fermentation of food and the production of volatile short-chain fatty acids (Sawabe et al. 2003; Sawabe 2006). The abalone hepatopancreas or digestive gland is a large secretory organ that produces digestive enzymes for extracellular digestion, but which can also absorb and engulf food processed in the crop and caecum for further metabolic transformations or intracellular digestion (Campbell 1965).

Abalone avoid high light intensities during the day and therefore graze more actively during the night, but they may also graze in sheltered areas during the day due to their slow feeding rate. In the South African abalone, *Haliotis midae*, digestive enzyme activities in the crop fluid coincide with the presence of food in the crop and are highest twelve hours after

the onset of feeding, approximately six hours after peak gut fullness (Britz et al. 1996). Abalone may graze on macroalgae, diatoms and detritus in the wild, whereas farmed abalone feed on formulated feed and diatoms growing in their tanks. South African abalone that occur in the Western Cape feed predominantly on brown macroalgae for which complex polysaccharides include alginate (1,4-linked β-D-mannuronic and α-L-guluronic acids), fucoidan (1,2-linked α-L-fucose monomers), cellulose (crystalline β-1,4-D-glucans) and laminarin (1,3-linked β-D-glucose monomers) (Haug et al. 1967; Lahaye & Kaeffer 1997; O'Sullivan et al. 2010) (see Chapter 1).

Abalone digestive enzymes that degrade carbohydrates from brown macroalgae may include alginate lyase (mannuronate lyase; Ostgaard & Larsen 1993), laminarinase (β-1,3-glucanase; Kumagai & Ojima 2009), fucoidanase (α-L-fucosidase) and cellulase (endo-β-1,4-D-glucanase; Suzuki et al. 2003). Since macroalgae are generally rich in complex polysaccharides (Erasmus et al. 1997), a range of other carbohydrases in abalone that degrade both alpha and beta-linked reserve and structural carbohydrates have been found; including, β-D-galactosidases, β-D-glucosidases and α-D-glucosidases (Bennet et al. 1971). Abalone enzymes also include α-amylase (Nikapitiya et al. 2009) which is probably employed in the digestion of microalgae, since microalgae are more readily digestible compared to macroalgae (Markou et al. 2012). In *Haliotis discus discus*, 30 % of genes from the hepatopancreas are dedicated to carbohydrate metabolism, whereas 18 % of genes are dedicated to protein metabolism and its biotransformations (Munasinghe et al. 2006). As a result, protease activities in abalone are relatively low compared to that of other invertebrates (Edwards & Condon 2001).

The activity levels of carbohydrases in abalone are influenced by diet type: *H. midae* fed brown macroalgae display relatively high levels of alginate lyase, laminarinase and cellulase activities compared to abalone fed red macroalgae (Erasmus et al. 1997). Digestive

enzyme activity levels in cultured abalone also change in response to a switch from macroalgae to formulated feed (Garcia-Esquivel & Felbeck 2006). Formulated feed, being relatively fibre-poor, contains less complex polysaccharides and high levels of reserve carbohydrates derived from grains, in addition to proteins derived from both animal and terrestrial plant sources. Farmed abalone can adjust well to formulated feeds when the shift occurs at a relatively early stage of development (Knauer et al. 1996) (see Chapter 2).

Abalone carbohydrases can originate from both endogenous and exogenous sources as bacteria isolated from the guts of abalone can produce enzymes that hydrolyse a range of different carbohydrates found in both macroalgae and formulated feed (Erasmus et al. 1997; Zhao et al. 2012). In *H. midae* and other abalone species that feed on brown macroalgae, alginate polysaccharides are fermented to short-chain fatty acids with the aid of symbiotic facultative anaerobic *Vibrio* bacteria (Sawabe et al. 1995; Sawabe et al. 1998; Sawabe et al. 2003) (see Chapter 1). Bacterial alginate lyases are usually specific for the guluronic acid blocks of the alginate hetero-polymer (Boyen et al. 1990; Sawabe 2006; Dong et al. 2012). Alginate lyase-excreting marine bacteria include those of the genera *Alginovibrio, Alteromonas, Pseudoalteromonas* and *Vibrio* (Wong et al. 2000). Since the presence of antibiotics in the abalone gut reduces the levels of macroalgal degrading enzymes in *H. midae*, it was suggested that marine bacteria play an important role in the digestion of macroalgal substrates (Erasmus et al. 1997). However, the role of bacteria in the digestion of formulated feed remains unknown.

In addition to carbohydrases, abalone also possess lipase, lysozyme and protease enzymes (Knauer et al. 1996; Garcia-Esquivel & Felbeck 2006), however, endogenous protease activity levels are relatively low compared to that of carbohydrases (Garcia-Esquivel & Felbeck 2006). Due to the relatively high content of protein in the formulated feed of cultured abalone, proteases in abalone have been well studied. The highest protease activities

for both *H. rubra* (pH 3) and *H. fulgens* (pH 2 – 5; 1.4 – 2.76 μmol tyrosine min^{-1} mg^{-1}) were found in digestive gland extracts and therefore the hepatopancreas may be an important site for protease secretion (Picos-García et al. 2000; Edwards & Condon 2001). Abalone proteases may have activity peaks at different pH values, reflecting different types of proteases which may also differ between species, but they seem to include both acid and serine proteases. Some proteases seem to retain efficient activity levels at physiological pH as active proteases within this range have been found in *Haliotis rufescens* at a pH around 5.5 (McLean 1970; Garcia-Esquivel & Felbeck 2006) and in *H. discus hannai* at pH 5.4 (Cho et al. 1983). Most abalone species seem to have acid protease activity which is probably due to the presence of aspartic proteases and cathepsins (Picos-García et al. 2000).

Alkaline proteases in abalone seem to be located in the lower regions of the digestive tract, which are also the most physiologically alkaline regions (Harris et al. 1998b). In wild adult *H. fulgens*, the highest levels of protease activity was found at alkaline pH (pH 10 – 11) in intestinal and rectal fluid extracts, followed by hepatopancreas extracts (pH 5) and crop-stomach content extracts at pH 7 (Serviere-Zaragoza et al. 1997). The authors noted that alkaline protease activity was due to the presence of trypsin and chymotrypsin, which was found only in the intestine and rectum, and chymotrypsin activity was ten times higher than that of trypsin. In contrast, juvenile *H. fulgens* displayed trypsin and chymotrypsin activities in both hepatopancreas (0.0027 – 0.011 and 0.008 – 0.016 in BAPNA and SAPNA units. mg^{-1} protein, respectively) and digestive tract (0.003 – 0.007 and 0.003 – 0.011 in BAPNA and SAPNA units. mg^{-1} protein, respectively) extracts (Picos-García et al. 2000).

Protease activity also varies in response to diet as higher protease activity was found in kelp-fed abalone compared to those fed formulated feed (Garcia-Esquivel & Felbeck 2006). It seems that protease activity levels respond to differences in diet quality, where protein in formulated feed would have a higher bioavailability than protein in macroalgae, for which

utilisation of macroalgal protein would require an increase in enzyme-facilitated digestion. Macroalgal diets may also promote higher numbers of naturally-occurring gut bacteria in abalone that can efficiently utilise the protein in macroalgae (see Chapter 2) through a specific suite of proteases. This would explain why both macroalgae diets and host-derived probiotic supplementation leads to increases in protease activity levels (Macey & Coyne 2005; Garcia-Esquivel & Felbeck 2006). On the other hand, dietary increases in reserve carbohydrate and protein concentration may increase the number of opportunistic gut bacteria in abalone fed formulated feed, which could lead to bacterial competition with the host for nutrients instead of symbiosis characterised by exogenous enzyme supplementation (see Chapter 1).

To identify bacterial enzyme activity, *in vitro* studies on the enzyme activities of bacteria isolated from the guts of abalone have been performed (Erasmus et al. 1997; Zhao et al. 2012). However, less than 10 % of abalone gut bacteria can currently be cultured (Tanaka et al. 2003; Tanaka et al. 2004) and therefore analysis of digestive enzymes produced by culturable bacterial isolates would not give a good representation of the total bacterial enzymes present in the host gut. The use of antibiotics in aquatic species have also been employed to directly compare digestive enzyme activity levels in the gut homogenates of antibiotic-treated animals, with diminished or absent gut-bacterial populations, to that of untreated animals (Erasmus et al. 1997; Xue et al. 1999). However, to effectively reduce gut-bacterial populations in aquatic animals without toxicity requires knowledge of the target bacteria and dose-response trials to antibiotics on the host animal. It was also found that the production of gnotobiotic abalone achieved by Erasmus et al. (1997) could not be reproduced in another lab for another abalone species (Enriquez et al. 2001). Enriquez et al. (2001) suggested that bacterial enzymes may still be active in the abalone gut after bacterial cells are lysed by bactericidal antibiotics.

In a preliminary experiment during the present study, treatment of abalone with antibiotics specifically targeted to the expected gut bacteria, following the specifications of the experimental setup used by (Erasmus 1996), resulted in a lack of feeding behaviour in antibiotic-treated abalone, which would therefore not have displayed normal enzyme activities. To avoid the number of variables that can interfere with enzyme activity comparisons when antibiotics are used to eliminate enzyme-producing gut bacteria, new technologies for directly identifying enzyme proteins and their origin can be used instead. Through the use of mass spectrometry and proteomic techniques (Holland et al. 2010; Fagerquist et al. 2014), functional proteins present in any given sample, and the bacteria or animal they originate from, can be identified. Through the use of these techniques digestive enzymes originating from both abalone and exogenous sources can be identified within the same gut sample by comparing identified proteins to that of a protein database. In addition, proteomic identification of proteins that can be matched with database proteins of specific bacteria may provide additional insights in the functional roles of specific bacterial types present in the abalone gut.

The hypothesis of the present study was that kelp supplementation in a formulated feed may result in a measurable shift in the gut microbial profile (see Chapter 5), through prebiotic or antibacterial mechanisms, and subsequent possible differential digestive enzyme activity levels in the guts of abalone fed kelp-supplemented (KS) and control diets. A preliminary 24 h sampling trial was undertaken to determine the time of peak enzyme activity for different gut sections and to establish the presence and type of bacterial derived digestive enzymes present in the guts of abalone fed a kelp-supplemented formulated feed. The results of the preliminary trial were used to design the sampling protocol for the comparison of enzyme activity in KS and control diet fed abalone.

METHODS

Experimental design

Commercially reared sub-adult abalone were used for a seven-week feeding trial for which abalone were fed the KS diet (a formulated feed supplemented with 0.88 % kelp) and a control diet (see Chapter 3 "Methods"). Prior to the feeding trial, experimental animals had been weaned onto formulated feed and fed a kelp-supplemented diet for approximately twelve months. Abalone baskets were stocked within a single tank on a commercial abalone farm (see Chapter 3 "Methods") with three replicate baskets per diet, arranged within the tank according to a randomised block design. The two experimental isonitrogenous and isoenergetic formulated diets were the same as those used in Chapter 3 and included a kelp-supplemented formulated feed ("KS diet", Chapter 3) and a non-supplemented control feed ("control diet", Chapter 3) which were similar in nutrient composition except for the presence of kelp in the KS diet. Abalone used for digestive enzyme comparisons between those fed the KS and control diets were collected seven weeks into a feeding trial (see below). During the trial, feed was supplied *ad libitum* once daily between 15:00 – 16:00 based on the amount of feed remaining on the feeder plate from the previous night as per standard farm practise.

24 h Sampling trial

For the preliminary 24 h sampling trial, commercially reared abalone fed the KS diet, which is one of the treatment groups used for enzyme comparisons between abalone fed different diets (see below), were sampled. Farmed abalone (39.04 ± 9.5 g, mean ± 1 S.D., ~60 mm shell length), which were fed the KS diet for approximately 14 months up until the evening prior to sample collection, were collected at: six hours (01:00), twelve hours (07:00) and 18 hours (13:00) after light intensity on the farm changed from daylight to that of twilight. This time of dusk was taken as the time for the onset of feeding. Samples were collected from

three basket replicates (N = 9 per sampling time; i.e. 3 abalone per basket replicate) for which abalone baskets were left undisturbed until six hours after the onset of feeding; when the first sampling event from abalone baskets (and subsequent samplings) was conducted randomly and quickly to cause minimal stress and disturbance for the remaining abalone in the tanks. Abalone samples were immediately stored at -20 °C for 40 minutes until they were fully euthanized.

Digestive enzyme activity comparison between abalone fed KS and control diets

Abalone which were fed the KS diet (30.97 ± 6.57 g, mean ± 1 S.D., ~55 mm shell length) and control diet (25.28 ± 7.36 g, ~52 mm shell length) for seven weeks up until the evening prior to sample collection, were collected twelve hours after the onset of feeding at 08:00 in the morning, from three basket replicates per diet (N = 6 per diet; i.e. 2 abalone per replicate basket). Abalone were immediately stored at -20 °C for 40 minutes until they were fully euthanized after which they were dissected.

Sample preparation

Abalone collected for both the preliminary study and for the digestive enzyme comparison between diets were dissected aseptically on ice and digestive tract fractions (see below) were homogenised with a mortar and pestle after cutting samples into small fragments using a sterilised scalpel. To each homogenised digestive tract fraction, chilled citric acid buffer (pH 5.2) was added and samples were subsequently centrifuged at 4 °C for 45 minutes at 18 000 rpm (JA 20) to obtain soluble enzyme extracts. Aliquots of the supernatant (enzyme extracts) were stored at -20 °C and 4 °C for short-term storage between experiments.

24 h sample series

For each time sample (N = 9) for abalone fed the KS diet, the digestive organs of three abalone were pooled with three replicates per sample for three different gut extracts: the

hepatopancreas, crop contents and the gastrointestinal lining. Three pooled samples of three abalone each were used so that spectrophotometric readings for all organ extracts for different times of sampling could be performed within the same assay. The hepatopancreas ("HEP") extract comprised of a specific apical section of the hepatopancreas which forms the anterior end of the hepatopancreas (Erasmus et al. 1997), whereas the crop ("CROP") slurry extract comprised of the crop contents, and the digestive tract "lining" ("LIN") extract comprised the whole length of the digestive tract (oesophagus, crop, stomach, style sac, intestines), scraped and rinsed free of gut contents and hepatopancreas tissue. Apical parts of digestive glands were dissected separately from other organs, and the crop was punctured to obtain the slurry (Britz et al. 1996) prior to dissection of the gastrointestinal lining. To each pooled homogenized digestive tract section sample, 10 ml of cold citric acid buffer (pH 5.2) was added prior to centrifugation.

Samples for enzyme comparison between diets

For abalone fed either the KS diet or the control diet, abalone gut "lining" samples (sectioned as described above) were individually assayed so that each diet was represented by six replicates. To each homogenized digestive tract "lining" sample, 2 – 3 ml cold citric acid buffer (pH 5.2) was added prior to centrifugation. The "lining" sections of the digestive tract, which comprises the abalone viscera (Picos-García et al. 2000), was chosen for these analyses in order to cover the basal enzyme activity of the whole digestive tract, for which resident bacteria and enzymes may be located along different parts of the digestive tract.

Establishing enzyme assay protocols with commercial enzymes

All reagents and chemical products for this study were obtained from Sigma-Aldrich®. Commercial enzymes including alginate lyase (Sigma product: A1603), D-glucanase (67138 *SIGMA*, for laminarinase), α-amylase (A3176), pepsin (P7000), trypsin (T1426) and

chymotrypsin (C4129) were used to establish and optimize enzyme assay protocols for the screening of different enzymes in abalone extracts. For carbohydrases, the production of reducing sugars was tested for assay conditions at room temperature at either a relatively acidic pH (pH 5.2) that would correspond with that of the abalone gut, or a neutral pH (pH 6.9 -7). Assays were allowed to run for 30 minutes and potato starch, sodium alginate (Sigma product: W201502) and laminarin (L9634) were used as substrates to test for α-amylase, alginate lyase and D-glucanase activities, respectively, using 1 % (w/v) substrate for each enzyme. For protease activity with haemoglobin (H7379, 1 % w/v substrate) substrate, the activity of pepsin was tested for at pH levels of pH 3, pH 4 and pH 5.2 at both 25 °C and 37 °C for ten minutes each. Trypsin and chymotrypsin activities were tested for with BAPNA (Na-Benzoyl-DL-Arginine 4-nitroanilide hydrochloride; B4875, 0.04 % substrate) and SAPNA (N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; S7388, 0.2 % substrate) as substrates for assays at pH 7.5 and 25 °C, ranging from five to ten minutes. Based on the activity of commercial enzymes at specific assay conditions, the assays were modified for experimental assays with extracts from the abalone gut. The same substrates were applied for experimental assays.

Establishing enzyme protein concentration

Soluble protein was determined for each sample in triplicate by the Bradford method (Bradford 1976), for which bovine serum albumin (BSA) in the concentration range of 0.2 – 0.8 mg. ml^{-1} was used to generate standard curves at 595 nm (R^2 = 0.993 and R^2 = 0.94 for 25 µl and 50 µl BSA volumes, respectively).

Enzyme assays

All enzyme assays were established using end-point colorimetric assays determined by a Powerwave X microplate reader spectrophotometer with KC Junior ™ software (Bio Tek®

Instruments, Winooski, VT 05404, United States). For each assay, the concentration of the end product (x), following incubation of enzyme extracts with substrates for a specific time period, was calculated from its absorbance at a specific wave length using standard curve equations ($y = mx + c$) plotted for concentration (x) against absorbance (y). Spectrophotometric readings for samples were performed in triplicate, using 96-well microplates (Greiner Bio-One, Kremsmünster, Austria). Enzyme specific activity (mg product. mg^{-1} protein) was expressed as the amount of end product produced per protein in the assay for a given time period, instead of using a rate to avoid the assumption that a linear relationship existed between the liberation of hydrolysis end products and time.

Statistica 12 was used to calculate normality tests and homogeneity of variances using the Shapiro-Wilk test and Levene's test, respectively, and to compare enzyme specific activities between different time and digestive tract section samples and between diets using Kruskal-Wallis ANOVAs and Mann-Whitney U tests, respectively.

24 h sample series

Carbohydrases

Enzyme activity for HEP, CROP and LIN samples at 6 h, 12 h and 18 h after the onset of feeding was established by measuring the concentration of reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller 1959), measuring absorption of DNS-bound reducing sugars at 540 nm. Standard curves were generated using glucose ($R^2 = 0.998$, amylase and laminarinase) and β-D-mannuronic acid ($R^2 = 0.998$, alginate lyase) in the concentration range of 0.2 – 1.0 mg. ml^{-1}. Enzyme activity reaction mixtures contained 1 % (w/v) of the substrates potato starch, laminarin and sodium alginate for amylase (pH 6.9), laminarinase (pH 5.2) and alginate lyase (pH 6.9) assays, respectively. The reaction volumes for all assays were 400 µl made up of 275 µl buffer (citric acid buffer for pH 5.2 and a

phosphate buffer for pH 6.9), 25 µl crude enzyme extracts and 100 µl substrate (11.43 mg. ml^{-1}).

For each assay, an enzyme control containing 25 µl enzyme and no substrate and a substrate control containing 100 µl substrate and no enzyme were made up to equal volumes to the assay mixtures by adjusting the buffer volumes. Assay mixtures were incubated at room temperature (18 – 24 °C) for 30 min, after which they were centrifuged for 5 min at 13 000 x *g* to separate undigested substrate from the supernatant. Supernatant fractions were subsequently mixed with a 3,5 dinitrosalicylic acid (DNS) colour reagent using a 1:2 ratio, boiled for 5 minutes and cooled. Of the boiled colour-reagent-reducing sugar mixtures, 250 µl was transferred to microplates and spectrophotometric readings were obtained at 540 nm.

Proteases

General protease activities for HEP, CROP and LIN samples at 6 h, 12 h and 18 h after the onset of feeding was established using a modified version of the Anson method (Anson & Mirsky 1932; Walter 1984) with incubation temperatures of 37 °C. Protease activity was measured as the concentration of tyrosine liberated from a haemoglobin substrate in ten minutes. A standard curve was prepared with tyrosine in the concentration range of 0.001 – 0.01 mg. ml^{-1} ($R^2 = 0.983$). For each assay mixture, 250 µl of a buffered haemoglobin (pH 7) substrate was incubated with 50 µl crude enzyme extract at pH 7. The reactions were terminated with the addition of 500 µl of 5 % trichloroacetic acid (TCA). For control samples, TCA was added before the enzyme extract. Reaction mixtures were centrifuged for 10 min at 10 000 x *g*, and 50 µl of the supernatant was transferred to a microplate to which 100 µl of 0.5 M NaOH was added in addition to 30 µl Folin-Coicalteau colour reagent and incubated for 15 min before measuring the absorbance at 578 nm. Specific activity was calculated as the amount (mg) of tyrosine produced in 10 minutes per mg protein in the assay.

Samples for enzyme comparison between diets

Carbohydrases

Enzyme activity for lining samples of abalone fed the KS and control diets was established by measuring the concentration of reducing sugars formed in a DNS assay as described above. Standard curves were generated using glucose ($R^2 = 0.998$, amylase and laminarinase), β-D-mannuronic acid ($R^2 = 0.998$, alginate lyase) and L-Fucose ($R^2 = 0.998$; fucoidanase) as standards in the concentration range of 0.2 – 1.0 mg. mL^{-1}. The enzyme assay reaction conditions were the same as above.

Proteases

Acid protease activity for lining samples of abalone fed the KS and control diets was established using a modified version of the Anson method (Anson & Mirsky 1932; Walter 1984) to establish the concentration of tyrosine liberated from an acidified haemoglobin (pH 3) substrate in ten minutes at 37 °C. For each assay mixture, 250 µl of acidified haemoglobin was incubated with 50 µl crude enzyme extract and the reactions were terminated with the addition of 500 µl of 5 % TCA, which was added before the enzyme extract for control samples. A standard curve was prepared with tyrosine in the concentration range of 0.01 – 0.1 mg. ml^{-1} ($R^2 = 0.984$). Reaction mixtures were centrifuged and the supernatant was allowed to react with a Folin-Coicalteau colour reagent as above and the absorbance readings were recorded at 578 nm. Specific activity was calculated as the amount (mg) of tyrosine produced in 10 minutes per mg protein in the assay.

Serine proteases

Trypsin and chymotrypsin activities were established according to the method of Serviere-Zaragoza et al. (1997). To establish trypsin activity for gut lining samples, a 0.5 % (w/v) BAPNA substrate was prepared in a 50 mM Tris-HCl buffer (with 20 mM $CaCl_2$). To 225 µl

BAPNA in Tris buffer (pH 7.5), 25 µl enzyme extract was added and the reaction was run for 10 minutes at 25 °C, after which it was terminated by adding 30 % acetic acid and at which point absorbance was recorded against a water blank at 410 nm. Trypsin activity was expressed in BAPNA units per mg as: ($A_{410\,nm}$/min x 1000 x volume of the reaction mixture)/(8800 x mg protein in the assay), where 8800 is the extinction coefficient of *p*-nitroaniline (Erlanger et al. 1961). Chymotrypsin activity was established by preparing a 0.5 % (w/v) SAPNA substrate in the same buffer, for which 25 µl enzyme extract was added to 225 µl of the buffered substrate (pH 7.5). The reaction was run for five minutes at 25 °C after which it was terminated with 30 % acetic acid and read against a water blank at 410 nm. Chymotrypsin activity was expressed as SAPNA units per mg as: ($A_{410\,nm}$/min x 1000 x volume of the reaction mixture)/(8800 x mg protein in the assay), where 8800 is the extinction coefficient of *p*-nitroaniline (Erlanger et al. 1961).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: sub-samples of different organ and dietary samples

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970). Two 0.5 mm thick 12 % acrylamide resolving gels (7 x 10 cm) containing SDS were prepared with a Tris-HCl buffer (pH 8.8), and ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added to induce polymerization (setting). The gels were set using a gel setting kit (Bio-rad Laboratories, Inc., California, USA) and levelled with absolute isopropanol which was subsequently dried off prior to piling of the stacking gels (3 x 10 cm). Stacking gels (4 % acrylamide) contained SDS and were prepared with a Tris-HCl buffer (pH 6.8), APS and TEMED. A 10-well comb was inserted into the stacking gel prior to setting to allow vertical electrophoresis from the wells to the bottom (~1cm from the bottom) of the resolving gel.

The gels were assembled in a Mini-PROTEAN® II electrophoresis chamber (Bio-rad Laboratories, Inc.) which was filled with a Tris-glycine SDS-PAGE running buffer (pH 8.3).

A Tris-HCl sample buffer (pH 6.8) which contained glycerol, SDS and bromophenol-blue was prepared and mercaptoethanol was added to a 1:19 ratio prior to the addition of sample extracts. Depending on protein concentration, two of each of the HEP, CROP and LIN 24 h time samples (collected 07:00) and three of each of the KS diet and control diet dietary samples were added to sample buffer in different ratios and were boiled for 5 minutes prior to loading the gel wells with 2 – 6 µl of the sample-buffer mixture. A molecular weight marker (3 µl; 14 – 116 kDa Pierce™ unstained protein molecular weight marker, ThermoFisher Scientific Inc., Waltham, MA USA) was added to one well of each gel. Electrophoresis was conducted at a constant voltage of 100 V for 30 – 90 min.

Gels containing separated proteins were stained and fixed with a Coomassie Blue solution containing methanol and glacial acetic acid. Gels were de-stained with a methanol-glacial acetic acid solution and viewed with a ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc.). Protein band patterns were determined through visual observations.

Proteomic classification of enzyme proteins in different organ samples

The three replicates of HEP and CROP 24 h time samples (for abalone fed the KS diet, collected at 07:00) were pooled to yield pooled HEP and CROP samples for proteomic analyses, containing a mix of proteins from nine abalone. Three gut lining samples from abalone fed the KS diet, which were used for dietary comparisons, were also pooled to yield a pooled "LIN" sample (N = 3 abalone). Due to financial constraints relating to the analyses costs of complex samples, sample analyses could only be performed for protein comparison between different sections of the digestive system (within abalone fed the KS diet), and not for between-diet comparisons. Therefore, the aim was to establish the presence and prominence of bacterial enzymes in the gut regions of farmed abalone fed kelp-supplemented

formulated feed while establishing the efficacy of the proteomic technique to identify digestive enzymes from abalone gut samples.

Samples were sent to the Proteomics unit at the Central Analytical Facility at the University of Stellenbosch. Sample proteins were extracted chemically and precipitated with acetone. Proteins were digested with trypsin (1 μg. μl^{-1}) for six hours at room temperature, followed by 16 hours at 37 °C. Protein digests were purified of reagents using a desalting technique. Liquid chromatography was performed on sample proteins using an Ultimate 3000 RSLC (Thermo Fisher Scientific Inc., USA) for which chromatography was performed at a constant flow rate at 50 °C. Mass spectrometry was performed using a Fusion mass spectrometer (Thermo Fisher Scientific Inc., USA) for which samples were run for 60 minutes.

RAW data files generated by the mass spectrometer were imported into Proteome Discoverer v1.4 (Thermo Fisher Scientific Inc., USA) and processed using Mascot and SequestHT algorithms for which searches against a decoy database were performed within a 1 % false discovery rate (FDR) threshold. Database searches were set within parameters which allowed for chemical modifications of protein extracts. Another database search was performed against the Uniprot database (2014 edition), using the X! Tandem Sledgehammer algorithm (01/09/2013). Species searches included searches against Vetigastropoda, alphaproteobacteria, gammaproteobacteria and epsilonproteobacteria. Output files were combined and viewed with Scaffold software v4.4.3. (Proteome Software, Inc., Portland, OR 97219, USA) which used a probability-based algorithm in Protein Prophet to validate protein identifications from different search engines. In Scaffold, the confidence level for viewing protein matches was set at 95 % for three peptides and SEQUEST XCorr scores (significant above 3.75) were used as a measure of significant spectra-database protein correlations. The

number of spectra matched per identified protein was normalised to look at the relative abundance of each protein for different gut sections.

RESULTS

Enzyme assays

24 h sample series

Carbohydrases

Amylase activity for abalone fed the KS diet displayed similar patterns for HEP, CROP and LIN extracts with amylases reaching particularly high activity levels in the morning, twelve hours after the onset of feeding (Figure 4.1). There were no significant pairwise differences in amylase activity between any of the gut sections for different times of sampling (Kruskal-Wallis ANOVA: $H_{8,27} = 17.58$, $p = 0.025$; $z < 2.78$, $p = 0.2 – 1$ for all pairwise comparisons).

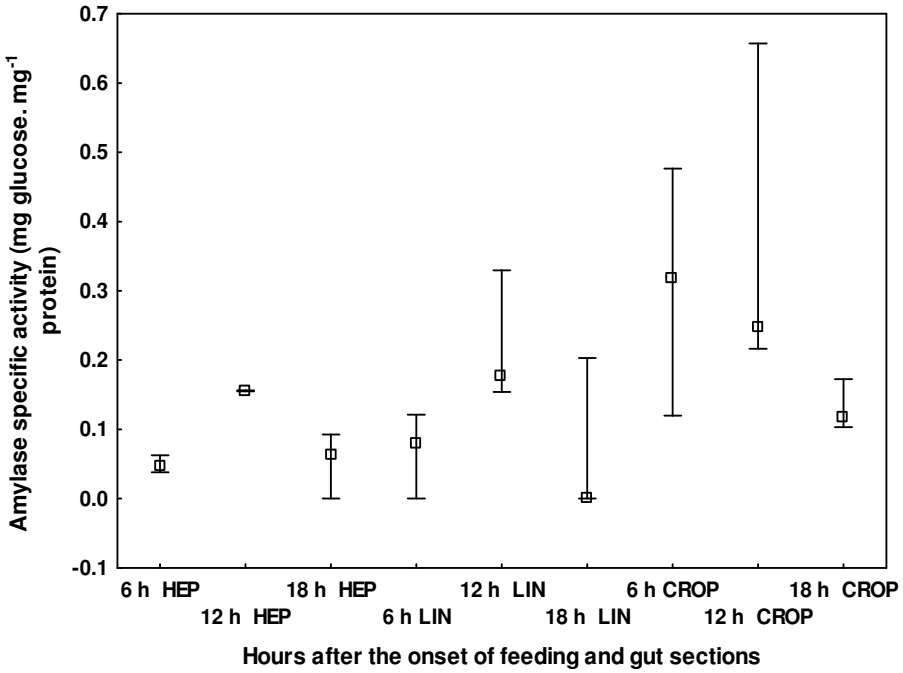

Figure 4.1: Medians (N = 3) with quartile ranges (whiskers) for amylase specific activity (mg glucose produced per mg protein in the assay) for enzyme extracts of different gut sections at different times after the onset of feeding for abalone fed the KS diet.

Alginate lyase activity for CROP samples at 12 h after the onset of feeding was significantly higher than that of HEP samples at 6 h and 18 h after feeding started ($z = 3.3$ and 3.5, $p < 0.03$), with no differences in pairwise comparisons between other samples ($z < 2.67$, $p = 0.27 - 1$) ($H_{8,27} = 22.15$, $p = 0.005$). Overall, alginate lyase displayed the same pattern as amylase activity for HEP and CROP extracts with highest activity levels in the morning, twelve hours after the onset of feeding and with highest overall activity found within the CROP extracts (Figure 4.2).

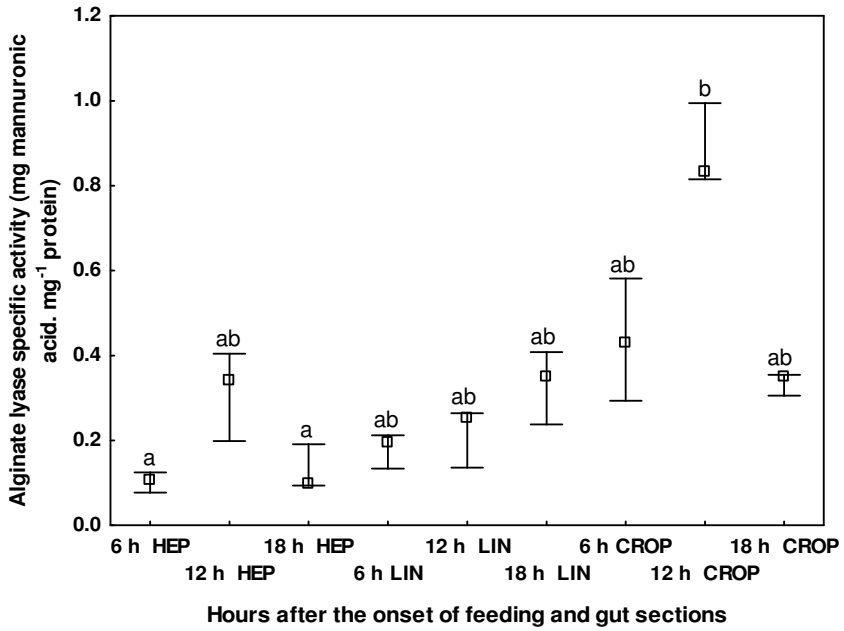

Figure 4.2: Medians (N = 3) with quartile ranges for alginate lyase specific activity (mg β-D mannuronic acid produced per mg protein in the assay) for enzyme extracts of different gut sections at different times after the onset of feeding for abalone fed the KS diet. Alphabetical letters that are different denote significant differences between samples ($p < 0.05$).

Laminarinase activity reached highest levels in CROP extracts with relatively low activity levels found for both HEP and LIN extracts, whereas highest activity in CROP extracts were found for abalone sampled in the morning, twelve hours after the onset of feeding (Figure 4.3). There were no significant differences in laminarinase activity between any of the gut sections for different times of sampling ($H_{8,27} = 18.26$, $p = 0.019$; $z < 2.83$, $p = 0.17 – 1$ for all pairwise comparisons).

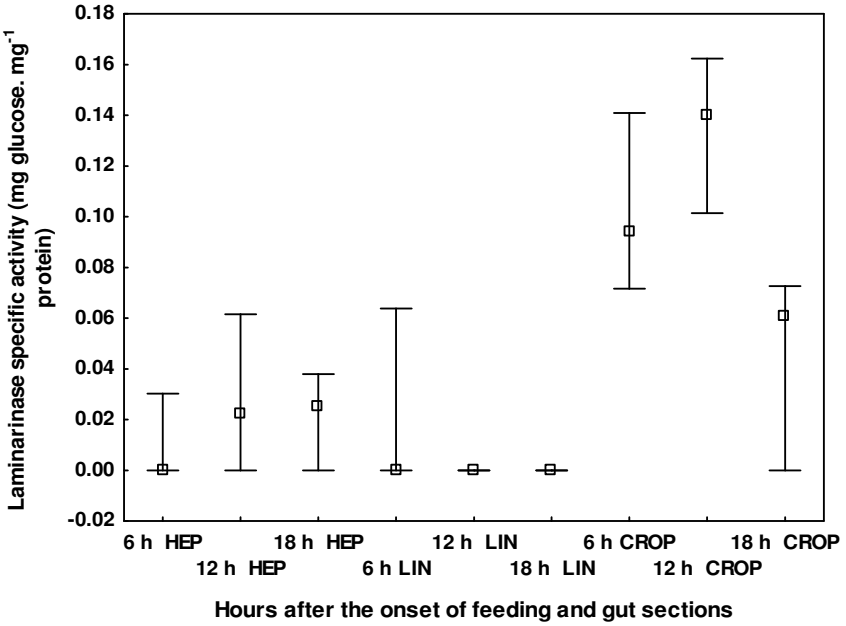

Figure 4.3: Medians (N = 3) with quartile ranges for laminarinase specific activity (mg glucose produced per mg protein in the assay) for enzyme extracts of different gut sections at different times after the onset of feeding for abalone fed the KS diet.

Protease

General protease activity was very low compared to the activity levels of carbohydrases found in this experiment. Proteases at neutral pH reached highest levels in HEP and CROP extracts 6 h and 18 h after the onset of feeding, respectively (Figure 4.4). There were no significant differences in protease activity between any of the gut sections for different times of sampling ($H_{8,27} = 13.3$, $p = 0.1$; $z < 2.57$, $p = 0.36 – 1$ for all pairwise comparisons).

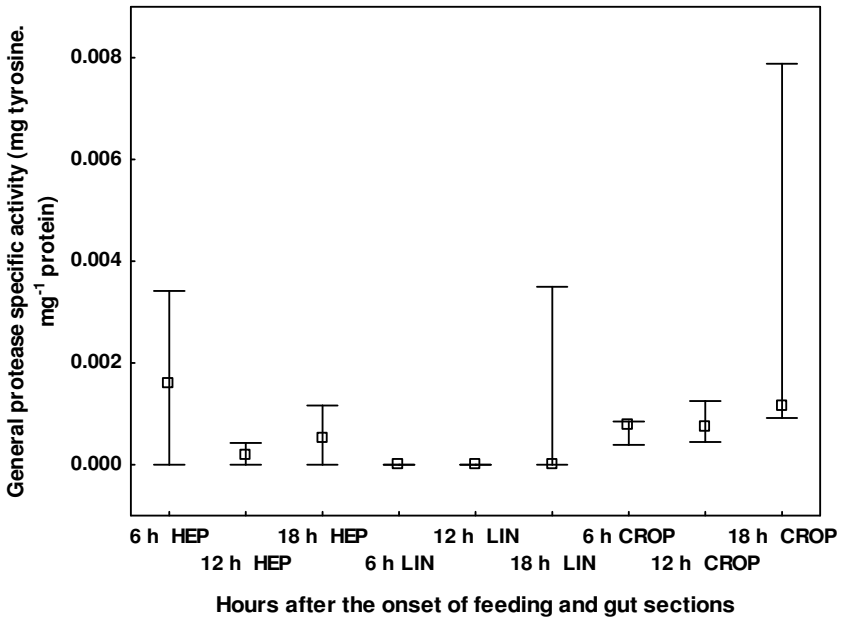

Figure 4.4: Medians (N = 3) with quartile ranges for general protease specific activity (mg tyrosine produced per mg protein in the assay) at neutral pH for enzyme extracts of different gut sections at different times after the onset of feeding for abalone fed the KS diet.

Enzyme activity comparison between abalone fed the KS and control diet

Carbohydrases

There were no significant differences in amylase activity between abalone fed the KS diet and control diet (Mann-Whitney $U_{6,6} = 13$, $z = -0.72$, $p = 0.48$; Figure 4.5). Similarly, there were no significant differences in alginate lyase (Figure 4.6), laminarinase (Figure 4.7) and fucoidanase (Figure 4.8) activity levels between abalone fed the KS diet and a control diet (M-W $U_{6,6} = 13$, $z = 0.72$, $p = 0.48$; M-W $U_{6,6} = 16$, $z = 0.24$, $p = 0.82$ and M-W $U_{6,6} = 18$, $z = -0.08$, $p = 1$, respectively). Carbohydrase enzyme activities were however less variable for abalone fed the KS diet in comparison to those of abalone fed the control diet.

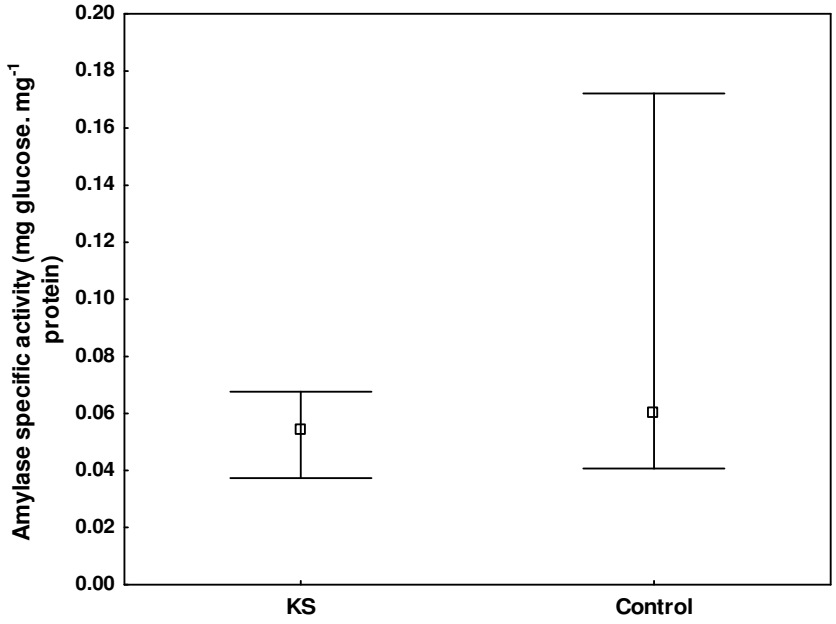

Figure 4.5: Medians with quartile ranges for amylase specific activity (mg glucose produced per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

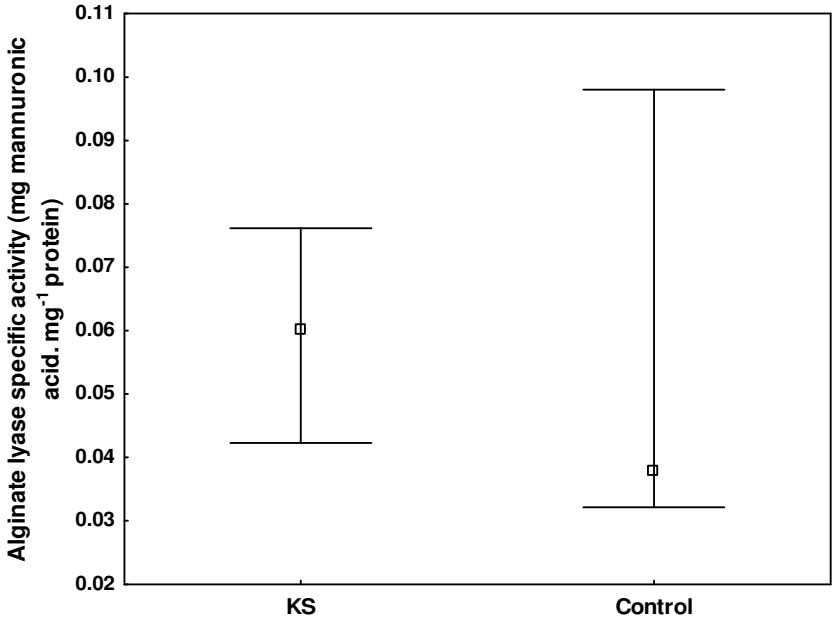

Figure 4.6: Medians with quartile ranges for alginate lyase specific activity (mg β-D mannuronic acid produced per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

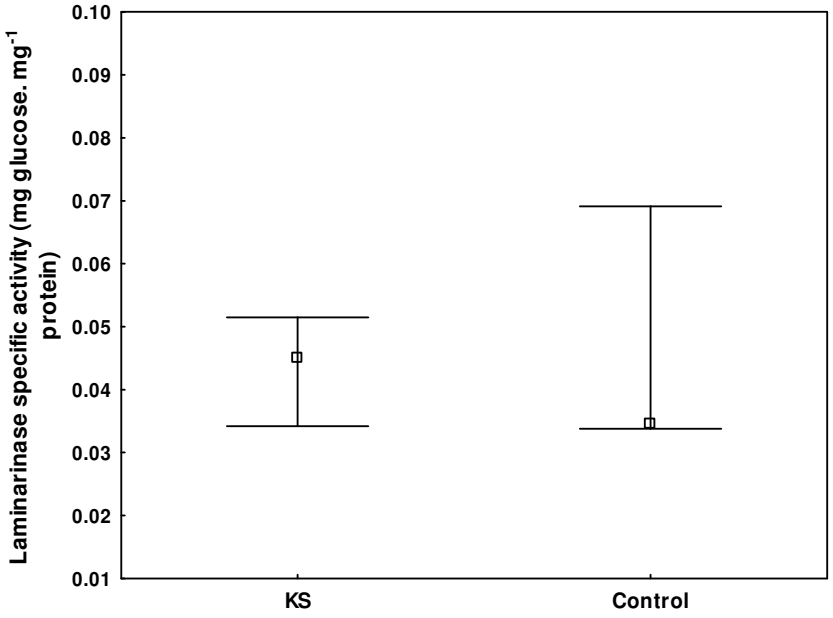

Figure 4.7: Medians with quartile ranges for laminarinase specific activity (mg glucose produced per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

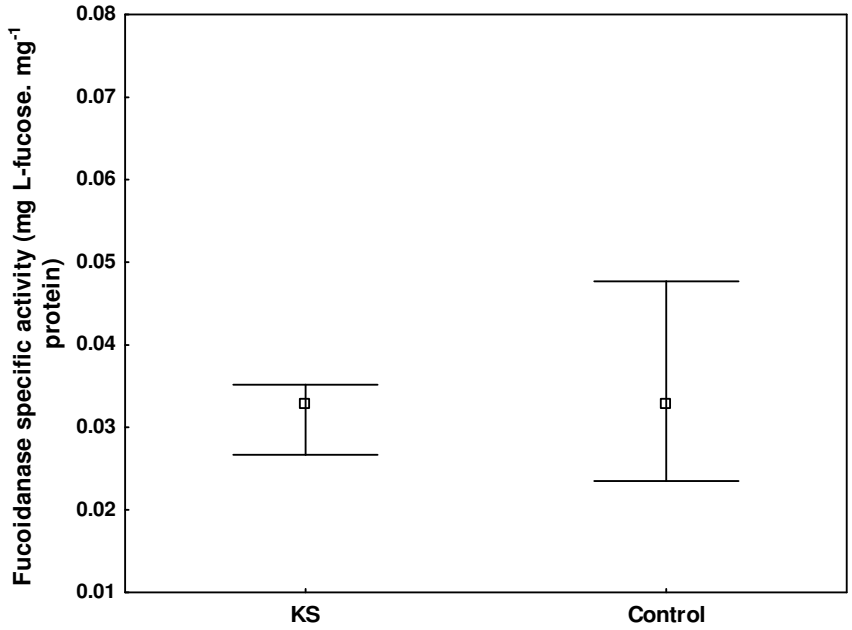

Figure 4.8: Medians with quartile ranges for fucoidanase specific activity (mg L-fucose produced per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

Acid protease

There were no significant differences in acid protease activities between abalone fed the KS and control diets (M-W $U_{6,6}$ = 12, z = -0.88, p = 0.39; Figure 4.9), but acid protease activity was less variable for abalone fed the KS diet in comparison to that of abalone fed the control diet.

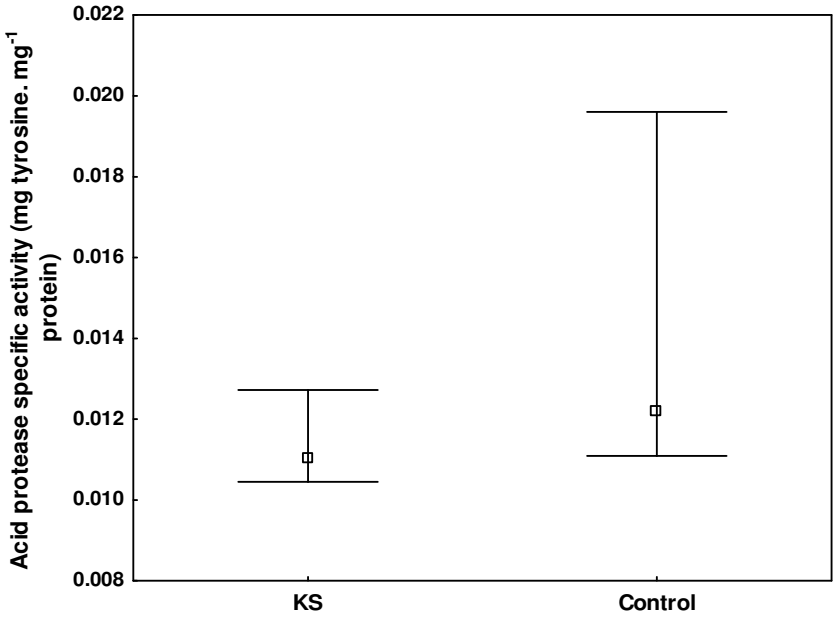

Figure 4.9: Medians with quartile ranges for acid protease specific activity (mg tyrosine produced per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

Serine proteases

Trypsin activity was not detectable for dietary samples of the digestive tract lining. Abalone fed the KS diet displayed higher chymotrypsin activities than abalone fed the control diet but this difference was not significant (M-W $U_{6,6}$ = 7, z = 1.68, p = 0.093; Figure 4.10).

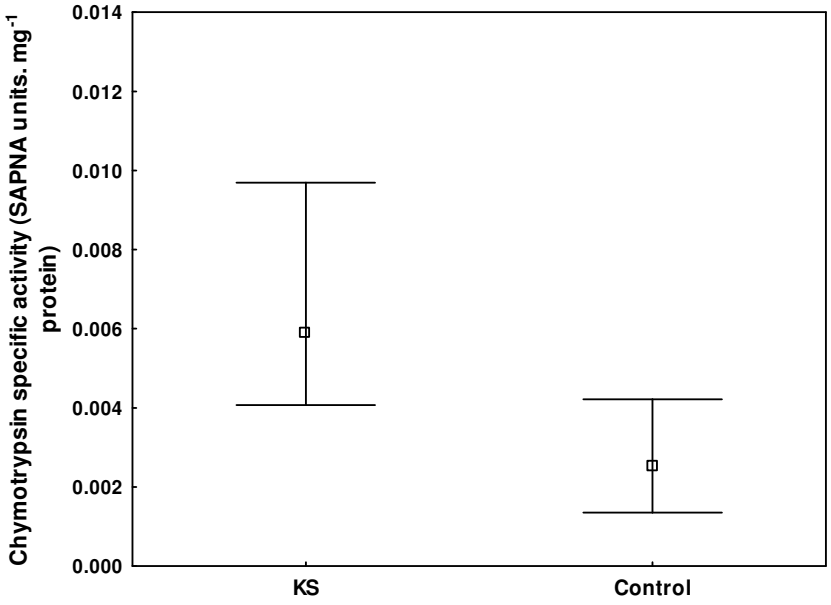

Figure 4.10: Medians with quartile ranges for chymotrypsin activity (SAPNA units per mg protein in the assay) for enzyme extracts of abalone fed the KS and the control diet (N = 6).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: sub-samples of different organ and dietary samples

HEP and LIN samples displayed similar protein band patterns (Figure 4.11) after electrophoresis through a polyacrylamide gel, but CROP samples displayed unique protein bands with a high molecular weight. For this gel the buffer level had to be maintained manually due to the use of older equipment which caused the gel to run slightly askew, but this would not have affected the separation of proteins. Protein band patterns for dietary samples were similar between samples for the control and KS diets (Figure 4.12), with prominent bands corresponding with the 66.2, 45.0 and 25.0 kDa bands of the protein marker.

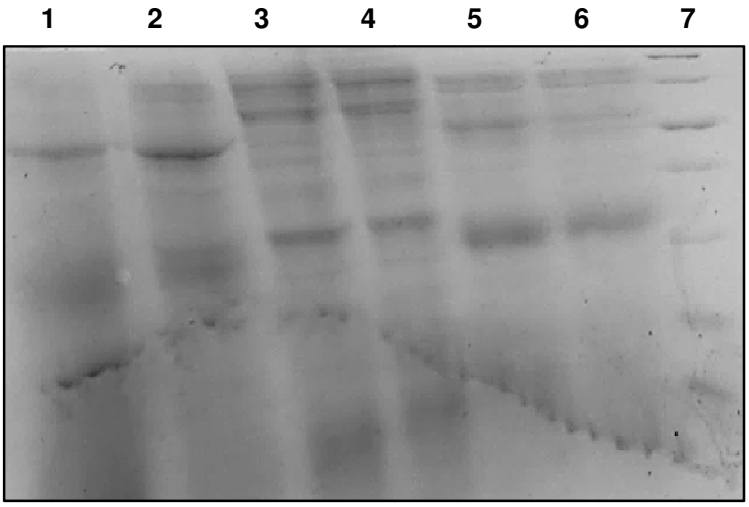

Figure 4.11: Separated protein bands are displayed for HEP (lanes 1 and 2), followed by CROP (lanes 3 and 4) and LIN samples (lanes 5 and 6) for 24 h time samples of abalone fed the KS diet. The molecular weight marker, starting at a 116 kDa for the top band followed by 66.2, 45, 35, 25, 18.4 and 14.4 kDa for the lowest band, is displayed in lane 7.

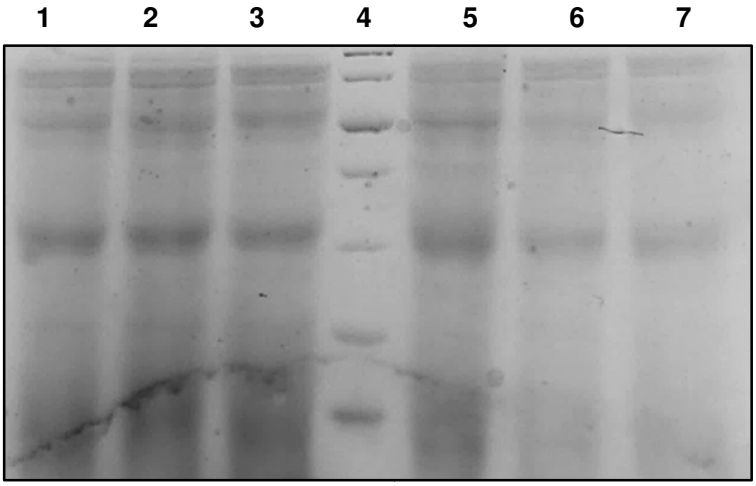

Figure 4.12: Separated protein bands are displayed for control diet samples (lanes 1, 2 and 3), followed by KS diet samples (lanes 5, 6 and 7). The molecular weight marker (same as above), is displayed in lane 4.

Proteomic classification of enzyme proteins in different organ samples

Among the digestive enzyme proteins that were identified to be of abalone origin for abalone fed the KS diet, were alginate lyase, cellulase (endoglucanases), α-amylase, laminarinase (β-1,3-glucanase), α-glucosidase, cathepsin B and a chymotrypsin-like serine protease (Table 4.1). Lysozyme was present in the CROP extract.

Table 4.1: Enzyme protein matches against the Vetigastropoda database. SEQUEST XCorr scores are all significant (> 3.75) and normalised spectrum counts for the number of spectra matches detected per protein reflect the abundance of each protein.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Species
Alginase	3.95 - 4.74	66.75			3.77 - 6.75	75.09	*Haliotis discus discus*
Alginate lyase 2 protein	4.24	34.19			3.77 - 6.12	34.72	*Haliotis rufescens*
α-amylase	4.27 - 4.73	13.02	3.62 - 4.54	1.74	3.81 - 5.84	19.38	*Haliotis discus discus*
α-amylase	3.81 - 4.38	26.05			3.88 - 4.83	16.15	*Haliotis tuberculata*
α-amylase					3.92 - 5.05	28.26	*Haliotis tuberculata*
β-1,3-glucanase	3.87 - 4.39	19.54			3.77 - 5.13	32.30	*Haliotis tuberculata*
Cathepsin B	4.68 - 6.12	92.80	3.77 - 5.42	62.76	3.75 - 6.06	12.92	*Haliotis discus hannai*
Cellulase	4.44 - 5.17	39.07	4.29	9.59	3.76 - 7.94	71.86	*Haliotis kamtschatkana*
Chymotrypsin-like serine proteinase	3.88 - 4.37	3.26	4.50 - 5.37	6.10	4.41 - 6.53	10.50	*Haliotis rufescens*
C-type lysozyme					4.61	3.23	*Haliotis discus hannai*
Endo 1,4-β-D-glucanase 1			4.6	0.87	3.95 - 5.26	5.65	*Haliotis discus discus*
Endoglucanase	4.44 - 6.91	91.17	4.29 - 6.21	27.89	3.76 - 8.22	**176.02**	*Haliotis discus discus*
Endoglucanase	3.81 - 6.91	**110.71**	3.76 - 5.32	41.84	3.75 - 8.22	**207.51**	*Haliotis tuberculata*
Alpha-glucosidase	4.29	6.51			4.33 - 5.99	12.92	*Haliotis discus hannai*

Table 4.1 continued: Enzyme protein matches against the Vetigastropoda database.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Endo-beta 1-4 mannanase					4.2-5.26	4.04	*Haliotis discus discus*
Fructose-bisphosphate aldolase	5.44	9.77	4.14-5.81	20.92	3.8-4.2	5.65	*Haliotis diversicolor*
Fructose-bisphosphate aldolase	3.9-4.79	52.10	3.75-5.81	146.44	3.76-5.69	45.22	*Haliotis rufescens*
Glyceraldehyde-3-phosphate dehydrogenase	3.81-6.84	76.52	3.75-5.67	251.03	3.77-6.5	110.62	*Haliotis discus discus*
Malate dehydrogenase	3.89-4.95	35.82	3.77-5.02	78.45	3.77-6.35	46.83	*Haliotis discus discus*
Triosephosphate isomerase	4.06	1.63	3.77-5.46	24.41	3.83-6.4	11.30	*Haliotis rufescens*

Protein searches against the alphaproteobacteria database for digestive tract extracts of abalone fed the KS diet (Table 4.2) yielded metabolic pathway-related proteins which were mostly detected within the HEP extract at relatively low abundances. A lower diversity of proteins at relatively higher abundances was found in the CROP and LIN extracts.

Protein searches against the epsilonproteobacteria database for digestive tract extracts of abalone fed the KS diet (Table 4.3) yielded metabolic pathway-related proteins which were only detected within the HEP extract. Aldehyde oxidoreductase, which forms part of a metabolic pathway in diverse bacterial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014), was detected and matched with that of a sulphate-reducing, N_2-fixing bacteria of the *Desulfovibrio* genus (Madigan et al. 2015). Subunits of urease, which are also found in diverse microbial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014) and produces ammonia as an end-product, were also detected. Phosphate acyltransferase, which acts on fatty acid derivatives and is involved in the biosynthesis of secondary metabolites (Kanehisa & Goto 2000; Kanehisa et al. 2014) was detected.

Table 4.2**:** Enzyme protein matches against the alphaproteobacteria database. SEQUEST XCorr scores are all significant (> 3.75) and normalised spectrum counts for the number of spectra matches detected per protein reflect the abundance of each protein. Superscripts "H" indicates that proteins matching that of specific bacterial genera were found in the HEP extract, whereas superscripts for "C" and "L" indicates that proteins matched to that of bacteria of a specific genus were found in CROP and LIN extracts, respectively.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Agarase	5.30 - 5.89	1.29					*Alteromonas*
Lytic protease	5.29 - 6.44	1.29					*Lysobacter*
β-peptidyl aminopeptidase	4.86 - 6.45	3.02	5.11	3.67	5.59	2.46	*Sphingosinicella*
Fatty acid oxidation complex subunit	5.36 - 6.00	2.15					*Shewanella*
Glucan 1,4-α-maltotetraohydrolase	4.97 - 5.52	1.72	4.63	7.36	3.38 - 5.56	7.36 - 9.82	*Pelomonas*^{HCL}, *Pseudomonas*^{C}
Pectate lyase	4.91 - 6.33	1.72 - 2.16	4.23 - 4.31	7.34	5.60 - 5.80	2.45 - 4.90	*Pectobacterium*^{HCL}, *Dickeya*^{HC}
Serralysin C	5.40 - 6.43	2.16					*Dickeya*

Table 4.2 continued: Enzyme protein matches against the alphaproteobacteria database.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Succinyl-CoA ligase [ADP-forming] subunit β	5.02 - 6.82	1.29 - 4.74	4.28	3.67	5.69	2.46	*Campylobacter*^{H}, *Desulfatibacillum*^{H}, *Desulfococcus*^{HC}, *Syntrophobacter*^{HL}, *Zymomonas*^{H}

Table 4.3: Enzyme protein matches against the epsilonproteobacteria database. SEQUEST XCorr scores are all significant (> 3.75) and normalised spectrum counts for the number of spectra matches detected per protein reflect the abundance of each protein.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Aldehyde oxidoreductase	5.43 - 5.91	2.87					*Desulfovibrio*
							Anaeromyxobacter,
Phosphate acyltransferase	5.40 - 7.25	4.00 - 7.50					*Geobacter*
Urease subunit	5.51 - 6.57	6.32					*Helicobacter hepaticus*

Protein searches against the gammaproteobacteria database for digestive tract extracts of abalone fed the KS diet (Table 4.4) suggested the presence of proteins for enzymes involved in a diverse suite of metabolic pathways. Subunits for acetophenone carboxylase and sulfite reductase and malate dehydrogenase were represented in all gut sections, but were better represented within LIN and CROP extracts. Acetophenone carboxylase and malate dehydrogenase form part of metabolic pathways in diverse microbial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014) and their proteins matched that of *Aromatoleum* (order: Rhodocyclales) and *Idiomarina loihiensis*, respectively.

Table 4.4: Enzyme protein matches against the gammaproteobacteria database. SEQUEST XCorr scores are all significant (> 3.75) and normalised spectrum counts for the number of spectra matches detected per protein reflect the abundance of each protein. Superscripts "H" indicates that proteins matching that of specific bacterial genera were found in the HEP extract, whereas superscripts for "C" and "L" indicates that proteins matched to that of bacteria of a specific genus were found in CROP and LIN extracts, respectively.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Acetophenone carboxylase subunits	5.10 - 8.79	3.83 - 4.47	4.38 - 4.79	**13.24**	4.29 - 4.35	5.68	*Aromatoleum*^{HCL},
Aspartate-semialdehyde dehydrogenase	5.15 - 6.40	2.56 - 3.83			4.31 - 4.34	1.89 - 3.78	*Buchnera*^{C}, *Pseudomonas*^{HC}
Enolase	5.27 - 6.36	2.56					*Idiomarina loihiensis*
Malate dehydrogenase	5.09 - 5.87	7.03	3.83 - 4.66	**129.78**	3.89 - 4.36	3.78	*Idiomarina loihiensis*
Sulfite reductase subunits	5.24 - 5.76	3.19	4.54	5.3	4.31 - 4.35	**16.08 - 22.71**	*Desulfovibrio*

Table 4.4 continued: Enzyme protein matches against the gammaproteobacteria database.

	HEP		LIN		CROP		
Protein name	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	SEQUEST XCorr score	Normalised spectrum count	Genus
Urease subunits	5.04 - 7.86	1.91 - 13.24	3.91 - 6.51	2.65 - 13.24	4.29 - 4.36	1.89 - 9.46	***Pseudomonas***^{HCL}, *Teredinibacter*^{HC}, *Nitrosococcus*^{HCL}, *Proteus*^{HC}, *Pseudoalteromonas*^{HCL}, *Beijerinckia*^{HCL}, *Marinobacter*^{HL}, *Alcanivorax*^{HC}, *Anaeromyxobacter*^{HCL}, *Azoarcus*^{HC}

DISCUSSION

Comparison of enzyme activity responses to food (for abalone fed the KS diet) for different gut sections at different time intervals revealed that the crop displayed particularly high carbohydrase activity levels in response to the presence of food in the gut, twelve hours after the onset of feeding and approximately six hours after expected peak gut fullness (Britz et al. 1996). Peak enzyme activities may therefore be found in the crop for extracellular digestion of carbohydrates, and both the gut lining and the hepatopancreas could have displayed basal enzyme activities that increase slightly after peak gut fullness. Significantly higher alginate lyase activities in the crop compared to that of hepatopancreas and gut lining sections may indicate that kelp components are digested mainly in the crop.

Proteomic classification of proteins in the gut for abalone fed the KS diet revealed that the abalone acid protease cathepsin B displayed a relatively low representation in the crop, and acid proteases were therefore mainly represented within the gut lining and hepatopancreas – where they may possibly be involved with intracellular digestion. An alkaline protease was mainly represented in crop and gut lining extracts. This study found relatively low *in vitro* protease activity levels, which is in agreement with that of other studies. As expected, proteins for endogenous polysaccharidases were well-represented within gut extracts, especially in the crop, and therefore abalone might be able to degrade most carbohydrates with the aid of endogenous enzymes.

Alginate lyases and α-amylase were relatively equally represented in the crop and the hepatopancreas extracts, whereas laminarinase, cellulases and "endoglucanases" were represented mostly in the crop extract, followed by the hepatopancreas extract. Cellulase and endoglucanases were also detected in the gut lining extract to a lesser extent. Endogenous endoglucanases in the crop may yield degradation end-products that provide substrates for bacterial metabolic pathways in the crop and the remainder of the digestive tract. Lysozyme

activity was also present in the crop extract and may play a role in controlling bacterial proliferation in this region.

Endogenous enzymes involved in intermediate reactions in metabolic pathways (anaplerotic reactions) were also well represented in the abalone digestive system and included endo-β-1-4 mannanase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase and triosephosphate isomerase. Most of these enzymes were well represented within different sections of the digestive system, but were especially well represented within gut lining extracts.

Enzymes that are involved in intermediate reactions in greater metabolic pathways and are responsible for the fermentation and conversions of metabolic pathway end-products and simple sugars were identified among the proteins of bacterial origin. Agarase, lytic protease (a serine endopeptidase) and serralysin C (an extracellular endopeptidase) were found for proteins matching those of *Alteromonas* (a marine bacterium), *Lysobacter* (an extracellular enzyme producing predator bacteria (Kobayashi et al. 2005; Madigan et al. 2015)) and *Dickeya* (an enteric glucose-fermenting bacterium) bacteria, respectively, in the hepatopancreas extract only. Fatty acid oxidation complex subunits matching that of a *Shewanella* bacterium, which associates with the outer surfaces of fish, were also only found in the hepatopancreas extract. β-peptidyl aminopeptidase, glucan 1,4-α-maltotetraohydrolase (a glycosidase), pectate lyase (a polysaccharidase) and subunits for succinyl-CoA ligase (present in diverse microbial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014)) were found in all gut extracts.

Malate dehydrogenase was particularly well represented within the gut lining extract and the correspondingly matched enzyme-producing bacteria, *Idiomarina loihiensis*, is believed to derive its energy mainly from the degradation of amino acids (Hou et al. 2004). Enolase, which forms part of metabolic pathways in diverse microbial communities

(Kanehisa & Goto 2000; Kanehisa et al. 2014) and which also matched database proteins for *Idiomarina loihiensis* was represented within the hepatopancreas extract only. Proteins for sulfite reductase, which had a relatively high occurrence in the crop, matched that of a *Desulfovibrio* species, which is a free-living anaerobic dinitrogen gas (N_2)-fixing bacterium (Madigan et al. 2015).

Aspartate-semialdehyde dehydrogenase, which forms part of metabolic pathways in diverse microbial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014) and was found in hepatopancreas and crop extracts, matched proteins from both *Buchnera* and *Pseudomonas* species. *Buchnera* species are endosymbionts in insects and *Pseudomonas* species are chemoorganotrophs that utilise a wide variety of organic molecules for energy sources (Madigan et al. 2015). The presence of subunits of urease, which forms part of metabolic pathways in diverse microbial communities (Kanehisa & Goto 2000; Kanehisa et al. 2014), were found to match urease proteins from many different bacteria, and different genera were represented in hepatopancreas, crop and gut lining extracts to different degrees. Genera included a high occurrence of the versatile chemoorganotroph *Pseudomonas*, N_2-fixing bacteria *Beijerinckia* and *Teredinibacter*, the ammonia-oxidizing marine bacteria *Nitrosococcus* and matches for *Pseudoalteromonas*. *Pseudoalteromonas* species are producers of many bioactive compounds and may be involved in important ecological roles (Bowman 2007).

The great diversity of bacterial enzymes in the gut extracts of abalone fed the KS diet suggests that they form part of numerous host and bacterial metabolic pathways and an intricate microbiome, which may interact with the abalone's metabolism. Hepatopancreas, crop and gut lining extracts varied in their composition of proteins as was suggested by gel electrophoresis, whereas proteomics suggested that the different gut extracts may differ in their composition of bacterial proteins. Most bacterial proteins which play a role in nutrient

acquisition were enzymes that are involved in metabolic pathways which follow from the degradation of complex food molecules into monomers or smaller organic compounds by the host. This implies that abalone gut bacteria play a different and possibly complementary role to that of the abalone host, which were the primary degraders of food macromolecules.

The presence of the kelp supplement in the KS diet was expected to affect the abalone gut microbiota and its ecology and therefore its associated enzymes made available to the host, but no differences in enzyme activity levels were found between abalone fed the supplemented diet and those fed the control diet. Since formulated feeds are designed to be digestible by abalone and to match their nutritional needs, the lack of differences in digestive enzyme activity levels might have been due to sufficient nutrient bioavailability in both diets due to a similar macronutrient composition and nutrient density. This would result in less undigested macronutrient substrates to be available for bacterial digestion. The results are in contrast to what would be found for diets that differ in quality and digestibility of nutrients, for which symbiotic associations would be found between microbes and hosts on a lower quality diet (Hungate 1975; Plante et al. 1990). In addition, diets with differences in nutrient bioavailability may stimulate compensatory adjustments in enzyme secretion (Clissold et al. 2010), as has been found in *H. rufescens* (~46 mm shell length), for which formulated feed resulted in higher cellulase activity and lower protease activity compared to kelp-fed abalone (Garcia-Esquivel & Felbeck 2006).

The diet history of experimental abalone on a kelp-supplemented diet and, prior to that, the same weaning diet (see Chapter 3 “Methods”), could have influenced the levels of endogenous enzymes in this study if the enzyme secretion patterns of abalone fed both diets were shaped by developmental effects. This would have caused most of the gene expression for digestive enzymes to have occurred at an early age, for which abalone fed the same diet would have established the same digestive physiology. Small abalone may therefore have a

higher flexibility to adjust enzyme levels in response to diet compared to sub-adult abalone (Bansemer et al. 2016). In addition, the core gut microbiome of experimental abalone may have been established prior to the trial during the time when both treatment groups were fed a kelp-supplemented diet. However, bacterial proliferation rates are rapid when an environment becomes favourable, and therefore the growth of opportunistic bacteria in the guts of abalone may have been influenced by diet and the resulting gut environment for sub-adult abalone in this study.

The introduction of the control diet for abalone in the present study may not have caused a significant shift in digestive enzyme activity levels compared to abalone fed the KS diet, but it did cause enzyme activities to be much more variable compared to that of abalone fed the KS diet. The higher variability in digestive enzyme levels found for abalone fed the control diet could reflect a gut environment that is less selective and therefore a less regulated gut-microbial community compared to that of abalone fed the KS diet (see Chapter 5) due to the absence of the macroalgal supplement which may harbour prebiotic-like or antimicrobial properties (see Chapter 1). This could result in an unstable microbial composition that constitutes both resident bacteria and opportunistic bacteria coupled with a variable microbial ecology. Therefore, a higher occurrence of transient opportunistic bacteria with variable resident times in the guts of abalone fed the control diet could result in a variable presence of specific bacterial enzymes. Variable gut microbial compositions could also affect the levels and types of organic compounds in the gut, and the gut-microbial ecology of abalone fed the control diet could be characterised by variable rates of bacterial conversions of monomeric sugar end products generated by abalone endogenous enzymes. This would cause variation in the amounts of reducing sugars that could be detected with spectrophotometric assays for gut samples of abalone fed the control diet.

The higher chymotrypsin-like activity in abalone fed the KS diet compared to those fed the control diet was not significant. However, a potential trend for increases in alkaline protease activity when abalone are fed the "potentially-prebiotic" KS diet was displayed. Such a trend would be in agreement with that found by Macey & Coyne (2005), who found an increase in alkaline protease activity in response to dietary probiotic supplementation. The bacterial enzyme responsible for the increased alkaline protease activity may not have been uncovered during this study since only a chymotrypsin-like enzyme of abalone origin was identified. However, many other bacterial-derived proteases and aminopeptidases could potentially have utilised the chymotrypsin substrate. This study detected chymotrypsin activity levels which were similar to that found by Picos-García et al. (2000), and the absence of trypsin activity may have been due to its much lower activity levels in abalone compared to that of chymotrypsin (Serviere-Zaragoza et al. 1997).

The degradation of sugars by bacteria is suggested by the presence of glucan 1,4-α-maltotetraohydrolase and pectate lyase, whereas utilisation of protein-derivatives by bacteria is suggested by the presence of β-peptidyl aminopeptidase and urease. The diversity of bacterial proteins found in the hepatopancreas, of which many were found exclusively in the hepatopancreas extract, was surprising since the hepatopancreas of *H. midae* is believed to be sterile (Erasmus et al. 1997). It is possible that many bacterial proteins may end up in the hepatopancreas due to the digestion of bacteria by lysozymes in the abalone crop, followed by absorption in the hepatopancreas.

In addition, intracellular digestion of food (or bacteria) may occur in cells of the hepatopancreas for which transport of particles for phagocytosis by the hepatopancreas may be facilitated by amoebocytes (Campbell 1965; McLean 1970). In primitive gastropod species such as abalone, extracellular digestion is supplemented by intracellular digestion through phagocytosis by amoebocytes and cells of the digestive gland (Owen 1966).

Alternatively, bacterial parasites may be present in the hepatopancreas to utilise the nutrients that are absorbed and digested in the hepatopancreas (Horwitz et al. 2016).

Many bacterial enzymes were distributed across the hepatopancreas, crop and gut lining extracts, but only malate dehydrogenase from the bacterium *Idiomarina loihiensis* was well represented within the gut lining extract, which may reflect the proliferation of this species along the digestive tract.

CONCLUSION

Since there were no differences in enzyme activity levels for enzymes involved in hydrolysing complex polysaccharides and protein molecules between abalone fed kelp-supplemented and non-supplemented feeds, it is suggested that abalone may degrade macronutrients in formulated feeds through their own suite of enzymes. However, the greater variation in digestive enzyme activity levels in abalone fed the control diet may indicate that kelp supplementation helps to regulate bacterial fermentation pathways of reduced sugars and other bacterial metabolic activities. Protein identification revealed that abalone endogenous enzymes involved in the degradation of macronutrients in formulated feed and kelp may be sufficiently present in the guts of abalone. The types of bacterial enzymes identified in this study, suggest that abalone gut bacteria may supply enzymes for intermediate reactions in metabolic pathways that follow from the degradation of larger food molecules to smaller compounds by abalone endogenous enzymes, and that they ferment sugars to fatty acids. This study presents a useful technique for characterising the functional roles of bacteria in the guts of abalone through establishing the presence of their proteins, while also defining the role of enzymes originating from the host for comparison with that of the bacterial enzymes.

CHAPTER 5

THE EFFECT OF LOW-LEVEL KELP SUPPLEMENTATION ON THE GUT-BACTERIAL COMMUNITY IN CULTURED ABALONE FED FORMULATED FEEDS

INTRODUCTION

The gut microbiota of wild abalone is hypothesised to play a role in the digestion of macroalgae (Erasmus et al. 1997; Sawabe 2006) due to their capacity to produce enzymes that can degrade macroalgal compounds (Erasmus et al. 1997; Zhao et al. 2012). In cultured abalone, dietary seaweed supplementation could act as a substrate for gut-bacterial growth and possible modes of action for bacterial modulation through seaweed supplementation in the guts of cultured abalone include prebiotic and bactericidal effects (see Chapters 1 and 4).

Several publications have identified the bacterial isolates from abalone reared on seaweed and formulated feed. In whole gut homogenates of 2-year-old *H. discus hannai* fed artificial feed containing seaweed powder, 85 % of isolated culturable bacteria were identified as facultative anaerobes, for which 62 % were identified as non-motile fermenters, and 20 % were identified as fermenters of the *Vibrio* genus (Tanaka et al. 2003). *Vibrio* species are symbionts of abalone that feed on brown-macroalgae and alginate-containing artificial feed, since they provide acetic acid as an energy source for abalone, whereas species like *Vibrio halioticoli* are able to convert polyguluronate to acetic acid after enzymatic degradation of alginate to di- and tri-saccharides by the abalone host (Sawabe 2006).

A diversity of alginate degrading bacteria were isolated from the gut of *H. gigantea* fed artificial feeds made of fish meal and *Laminaria*, for which genera included *Algibacter* and

Tenacibaculum (phylum: Bacteriodetes), *Roseobacter*, *Ruegeria* and *Silicibacter* (phylum: alphaproteobacteria), and *Agarivorans*, *Shewanella* and *Vibrio* (phylum: gammaproteobacteria) (Tanaka et al. 2015). More than 80 % of the isolated alginate-degrading bacteria belonged to the genus *Vibrio* and it was therefore suggested that alginate-degrading *Vibrios* are common in the guts of abalone (Tanaka et al. 2015). In contrast, bacteria isolated from different gut sections of adult Australasian abalone *H. laevigata* fed mixed macroalgae revealed mostly isolates from the family Enterobacteriaceae (phylum: gammaproteobacteria) (Harris et al. 1998b), which can utilise a wide range of carbohydrates as energy sources (Madigan et al. 2015). Australasian abalone may form different associations with bacteria compared to that of abalone that feed predominantly on brown macroalgae (see Chapter 1).

Easily-cultured bacteria that have been isolated from the guts of different marine invertebrates are similar between species and comprise mostly of *Vibrio* and *Pseudomonas* species for South African abalone (Erasmus et al. 1997), for the sea urchin *Echinus esculentus* (Unkles 1977) and for marine copepods and the green mussel *Perna viridis* (Sochard et al. 1979; Mohan et al. 1986). Bacterial isolates from the gut of the sea-cucumber *Holothuria atra* were also identified as *Vibrio* along with *Bacillus* (phylum: Firmicutes) and Actinomycetales (phylum: Actinobacteria) species (Ward-Rainey et al. 1996). However, culture-dependent analyses of animal gut microbes may be biased due to an over-representation of bacteria that grow easily on culture media. Culture-independent metagenomic analyses of abalone gut bacterial communities through DNA sequencing provide a better means than culture-dependent analyses to characterise the full spectrum of gut bacteria in abalone.

The relatively acidic abalone crop, stomach and style sac hosts a lower diversity of bacteria than the intestine (Harris et al. 1998b). Molecular identification of bacteria residing

in the abalone intestine has revealed that the intestinal bacterial community of cultured adult *H. diversicolor* comprised two dominant groups: the Proteobacteria and Tenericutes phyla (Huang et al. 2010). Dominant classes in terms of relative abundance included Mollicutes (phylum: Tenericutes, *Mycoplasma* related genera), followed by deltaproteobacteria and betaproteobacteria, whereas gammaproteobacteria, alphaproteobacteria and Verrucomicrobiae were present to a lesser extent (Huang et al. 2010).

Similarly, culture-independent DNA sequence analysis of the intestinal bacterial community of *H. diversicolor* revealed that gut-bacterial phyla, for abalone at different developmental phases, included a majority of gammaproteobacteria, followed by Firmicutes (class: Bacilli) (Zhao et al. 2012), whereas Firmicutes are closely related to the Tenericutes group found by Huang et al. (2010) (Madigan et al. 2015). Firmicutes were present in the guts of the non-feeding larval and early juvenile (diatom-feeding) abalone and gammaproteobacteria occurred within the guts of abalone of all developmental stages.

16S rRNA gene clone library analysis of abalone gut bacteria for whole gut samples revealed that gut bacteria of *H. discus hannai* comprised the same dominant phyla of Proteobacteria and Tenericutes compared to that of intestinal bacteria in *H. diversicolor* (Tanaka et al. 2004). Tanaka et al. (2004) found that the guts of juvenile *H. discus hannai* fed artificial feed comprised of alpha-, gamma- and epsilonproteobacteria and Mollicutes. The authors compared the gut-bacterial communities of abalone fed artificial feed to that of starved abalone or abalone fed brown macroalgae (*Laminaria*) and found that Mollicutes and Proteobacteria were present in all groups, but shifts in the most dominant phyla occurred for different groups. For abalone fed artificial feed, alphaproteobacteria (*Sphingomonas*) were most abundant, followed by Mollicutes, and gammaproteobacteria (*Vibrios*), and epsilonproteobacteria to a lesser extent. For abalone fed brown macroalgae, Firmicutes was the dominant phylum for which the majority were identified as Mollicutes, and

alphaproteobacteria (phylogenetically related to marine sponge symbiotic bacteria) were present to a lesser extent. The guts of starved abalone comprised a majority of Fusobacteria, followed by Mollicutes, alphaproteobacteria (Rhodobacterales) and gammaproteobacteria (Vibrionaceae).

A comparison of gut-bacterial diversity for whole gut samples of abalone fed either artificial feed or seaweed, has revealed that bacterial diversity was relatively high in abalone fed artificial feed compared to those that were starved or fed *Laminaria* (Tanaka et al. 2004). In combination with a relatively high gut-bacterial diversity, bacterial species dominance in abalone fed artificial feed was low compared to those fed seaweed (Tanaka et al. 2004). In contrast, bacterial diversity of intestinal samples, determined by the number of DNA bands analysed with DGGE analyses, for abalone fed artificial feed during their late juvenile phase was relatively low compared to abalone fed red seaweed during their grow-out phase (Zhao et al. 2012). However, the observed decrease in bacterial diversity observed for juvenile abalone fed artificial feed coincided with a diet switch from diatoms to artificial feed and it was therefore suggested that weaning abalone may be sensitive to bacterial-community disruption (Zhao et al. 2012).

In chapter two it was hypothesised that the enhanced growth in abalone fed a seaweed-supplemented formulated feed compared to a non-supplemented feed may be due to a shift in the bacterial community which enhanced digestive efficiency. Shifts in a species' gut microbiota in response to diet are often referred to as a gut-bacterial "modulation" which is defined as "the alteration in function or status of something in response to a stimulus or altered chemical or physical environment" (Taber 2013). Gut-bacterial community modulation in abalone have been achieved with the use of probiotics (Iehata et al. 2014), and in this chapter it is hypothesised that dietary kelp supplementation leads to a modulation of

abalone gut-bacterial communities through a prebiotic effect associated with the kelp substrate.

Published studies on Asian abalone species have characterised the composition of gut bacteria of abalone that are fed formulated feed containing seaweed-meal (Tanaka et al. 2003), but none have compared the gut bacterial communities of abalone fed formulated feed with and without seaweed supplementation. Therefore, the aim of this study was to compare the gut-bacterial communities of *Haliotis midae* reared on a formulated feed with and without kelp (*Ecklonia maxima*) inclusion. Abalone gut-bacterial communities were profiled in terms of relative abundances of bacterial phyla and bacterial diversity between cultured abalone fed a kelp-supplemented formulated feed and those fed a non-supplemented formulated feed.

METHODS

Sample collection

Abalone from the feeding trial described in Chapter 3 were used for gut-bacterial analyses. The two experimental isonitrogenous and isoenergetic formulated diets were the same diets used in Chapters 3 and 4: a kelp-supplemented formulated feed ("KS diet") and a non-supplemented control feed ("control diet") (see Chapter 3). To limit the presence of food-associated ingested bacteria in the gut during peak gut fullness, abalone fed both the KS diet (31.41 ± 10.45 g, mean ± 1 S.D.) and control diet (30.9 ± 6.64 g) for seven weeks, were starved for one day prior to sample collection and were collected the next morning from three basket replicates per diet (N = 7 – 8 per diet). Sampled abalone were immediately euthanized at -20 °C for 40 minutes.

Whole abalone were submerged in 70 % ethanol for one minute to remove surface-associated bacteria (Sawabe et al. 1995), and dissected aseptically on ice. The digestive tracts (the viscera), including the oesophagus, crop, stomach, style sac and intestines, scraped

free of the food slurry and the hepatopancreases, were homogenised together with a mortar and pestle after cutting samples into small fragments using a sterilised scalpel. Samples were stored at -20 °C prior to DNA extraction.

DNA extraction

DNA was extracted in duplicate for samples of individual abalone digestive tract extracts, with a PowerFecal® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA 92010, USA), and pooled. For each sample, 0.25 g of gut homogenate was added to microcentrifuge tubes containing small (~1 mm diameter) garnet beads, to which three volumes of lysis buffer was added. A SDS-detergent containing solution was added and the mix was heated to 65 °C to aid cell lysis and the breakdown of lipids and polysaccharides. The heated samples were subsequently vortexed to allow the beads to collide with cell walls in the presence of the cell lysis buffer. The tubes were centrifuged and the lysate was transferred to a new microcentrifuge tube and mixed with a reagent to precipitate organic and inorganic contaminants, after which the precipitate was separated from the lysate through centrifugation. The DNA-containing lysate was transferred to a spin filter to allow DNA to bind with a silica membrane in the presence of a high salt concentration for which the filtrate was discarded. The membrane-bound DNA was subsequently washed with an ethanol-containing solution. DNA was released from the membrane with the addition of an elution buffer within which the DNA suspension was stored at -20 °C.

Gel preparation for DNA viewing

To analyse the quality of the extracted genomic DNA and the size of the DNA fragments, gel electrophoresis was performed using 1 % agarose gels containing 1 mg/ml ethidium bromide. Polymerase chain reaction (PCR)-produced amplified DNA was also analysed with gel

electrophoresis to establish the reaction specificity of the PCR targeted for 16S rRNA **p**roducts and the size of the amplified DNA.

Pyrosequencing

Polymerase chain reaction protocol

DNA extracted from whole gut samples (3 – 17 ng. μl^{-1}) were amplified in a 25 µl PCR reaction, with 12.5 µl (1X) of a 2X KAPA HiFi HotStart ReadyMix® buffer (KAPA Biosystems, Massachusetts, USA) which contained a high fidelity DNA polymerase enzyme, 2.5 mM $MgCl_2$ and dNTP's, 0.75 µl of each of the forward and reverse primers (with 0.3 µM final concentration), and the sample DNA template (2 – 5 µl). The same primer pairs with different Multiplex Identifier Tags (MIDs) or barcodes were used for each sample to assign DNA sequences generated to their originating sample. The primer pair E517F (sequence: 5'-CAGCAGCCGCGGTAA-3') and E969-984 (5'-GTAAGGTTCYTCGCGT-3') for amplification of variable regions 4 and 5, corresponding to positions 517 – 533 and 969 – 984 of the *E. coli* 16S rRNA gene, was selected due to its high coverage of known bacterial phyla (Wang & Qian 2009; Matcher et al. 2011). The PCR thermal cycling parameters were optimized and are displayed in Table 5.1. The PCR annealing temperature was set at 50 °C and the thermal cycles were kept below 35 to reduce the specificity of the reaction for dominant bacterial species and to allow the DNA of less dominant species to be amplified. Due to the difficulty of the PCR reaction with the fusion primers which operate best with a very specific PCR protocol and DNA quality, only the PCR products with the highest quality i.e. four for each diet were selected for pyrosequencing. PCR products were purified and subjected to emulsion PCR, after which they were sequenced with a GS FLX Titanium Sequencer (454 Life Sciences, Roche) at the Department of Biochemistry and Microbiology at Rhodes University.

Table 5.1: Thermal cycling protocol for PCR DNA amplification for pyrosequencing.

Initial denaturation	98 °C	5 min	1 cycle
Denaturation	98 °C	45 s	
Annealing	50 °C	30 s	5 cycles
Extension	72 °C	1 min	
Denaturation	98 °C	45 s	
Annealing	50 °C	30 s	17 – 32 cycles
Extension	72 °C	1 min	
Final extension	72 °C	1 min	1 cycle

Data curation and analysis

The pyrosequencing facility at Rhodes University provided the raw sequence flow-gram files (SFF files) which were generated with 454 Life Sciences software. Sequences for these files were assigned to barcodes, quality-filtered, de-multiplexed and de-noised (to remove sequencing errors) using QIIME (Quantitative Insights Into Microbial Ecology) version 1.8.0 (Caporaso et al. 2011b). Python scripts were used for the remainder of the analyses in QIIME, for which QIIME was also used for the following: to filter out short reads with lengths that were below 200 base-pairs, to perform *de novo* clustering of sequences into operational taxonomic units (OTUs) using UCLUST (Edgar 2010) at a 97 % sequence similarity level, to assign taxonomies and representative sequences from clustering using the greengenes (August 2013 release) and Silva_111 databases, to align representative sequences against the greengenes database using PyNAST (Caporaso et al. 2010) (Caporaso et al. 2010a) and to remove chimeras from assigned OTUS using Chimera Slayer (Haas et al. 2011) and a python filter script. Alpha-rarefaction, Shannon diversity (H) and Simpson diversity analyses were also performed in QIIME. In QIIME, Shannon diversity is calculated as $H' = -\Sigma^{S}_{i=1} (p_i \ln(p_i))$, where pi = the proportion of individuals in the i^{th} species and S is the number of species observed. Simpson diversity is calculated as 1 – D; where $D = \Sigma\, n(n-1)\, /N$

(N-1), for which N = total number of individuals of all species and n = total number of individuals for each species.

Non-metric multidimensional scaling (n-MDS) and One-way-ANOSIM analyses were performed with PAST® statistical software (Hammer et al. 2001) using a Bray-Curtis similarity measure. Statistica 12 was used to calculate normality tests and homogeneity of variances using the Shapiro-Wilk test and Levene's test, respectively, and to compare the relative abundances of gut-bacterial phyla between abalone fed the KS diet and the control diet using Mann-Whitney U tests.

Denaturing gradient gel electrophoresis analysis

Polymerase chain reaction protocol

Extracted DNA from whole gut samples were amplified in a 25 µl PCR reaction, using a DreamTaq buffer and a DreamTaq DNA polymerase (ThermoFisher Scientific Inc., Waltham, MA USA), dNTP's, 2.5 µl of each of the forward and reverse primers, and the sample DNA template (5 µl). The primer pair consisted of universal primers E9F (5′-GAGTTTGATCCTGGCTCAG-3′) and U1510R (5′-GGTTACCTTGTTACGACTT-3′) (Maropola et al. 2015). A simple PCR protocol with initial denaturation at 94 °C for 4 min and 1 cycle, followed by; denaturation (94 °C for 30 s), annealing (52 °C for 30 s) and extension (72 °C for 105 s) for 30 – 35 cycles, and final extension at 72 °C for 10 min was used. The PCR products that were generated were used to conduct a nested-PCR to generate a smaller DNA product of about 600 nucleotide base pairs (bp) for which 1 µl was used as template in a 25 µl nested-PCR reaction. For the reaction, the DreamTaq buffer and a DreamTaq DNA polymerase (ThermoFisher Scientific Inc., Waltham, MA USA), dNTP's and 2.5 µl of each of the forward and reverse primers were used. The primer pair consisted of the universal primer 907R (5′-

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTTG

AGTTT-3′) with a GC clamp (underlined) and the bacterial primer GM5F (5′-CCTACGGGAGGCAGCAG-3′), corresponding to positions 907-928 and 341-357 on the *E. coli* 16S rRNA gene (Santegoeds et al. 1998). A touch-down PCR protocol was used (Santegoeds et al. 1998; Bowker 2002; Table 5.2). PCR products are displayed in Figure 5.1.

Table 5.2: Thermal cycling protocol for nested-PCR DNA amplification for DGGE analyses.

Initial denaturation	95 °C	2 min	1 cycle
Denaturation	94 °C	30 s	
Annealing	68 °C	45 s	4 cycles
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	
Annealing	66 °C	45 s	4 cycles
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	
Annealing	64 °C	45 s	4 cycles
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	
Annealing	62 °C	45 s	4 cycles
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	
Annealing	60 °C	45 s	4 cycles
Extension	72 °C	2 min	
Final extension	72 °C	5 min	1 cycle

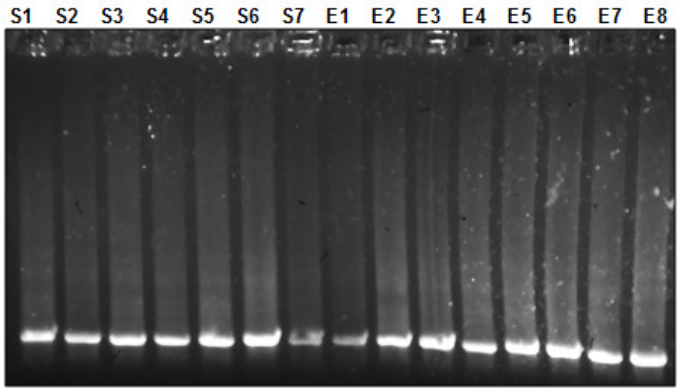

Figure 5.1: The ~600 base pair nested-PCR products used for DGGE analyses.

Electrophoresis

DGGE analysis was performed with the DCode™ Universal mutation detection system (Bio-Rad) with 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA at pH 8.3) as a running buffer for a 10 % 1 mm polyacrylamide gel. A denaturing gradient of 30 – 70 %, using a mixture of urea and formamide as denaturants, was used after initial optimization of the gradient. A volume of 20 µl of PCR products mixed with 10 µl loading dye was loaded into each of the gel wells. Electrophoresis was performed at 60 °C and a 100 V for sixteen hours. The DGGE gel was stained with ethidium bromide and photographed on a UV trans-illumination table with a built-in camera unit.

Data analysis

Gel bands were analysed as presence-absence data to conduct a n-MDS plot with PAST.

RESULTS

Pyrosequencing

Rarefaction curves for observed species showed that the majority of the eight samples (four samples for each of the KS and control diets) that were sequenced approached a plateau (Figure 5.2) and were therefore sampled to saturation of the community coverage (Hughes & Hellmann 2005; Matcher et al. 2011), despite some samples comprising of fewer sequences than others (Table 5.3). Shannon and Simpson diversity indices indicated that the most and least gut-bacterial diversities for individual abalone samples were found for abalone fed the control diet (Table 5.3). However, the two control diet samples which yielded the lowest diversities also contained the highest proportion of "unassigned" sequences that did not match any bacteria in the databases tested (Figure 5.3).

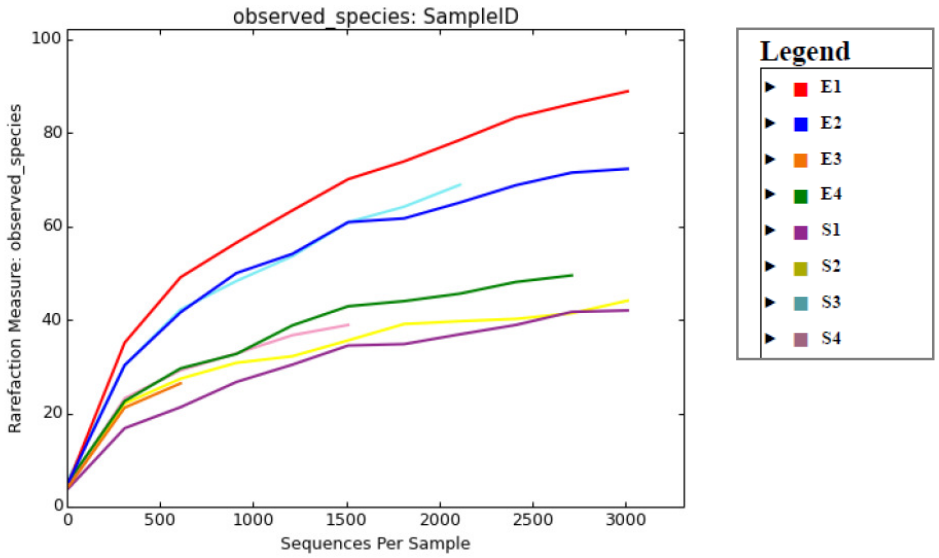

Figure 5.2: Rarefaction curves of the observed species per sample plotted against the number of sequences per sample for KS diet sample replicates ("E" samples) and control diet sample replicates ("S" samples).

Table 5.3: Means (± 1 S.D.) for number of sequences and species detected and diversity indices are displayed for KS and control diet samples.

	Control diets					**KS diets**				
	S1	**S2**	**S3**	**S4**	**Average ± S.E.**	**E1**	**E2**	**E3**	**E4**	**Average ± S.E.**
# of reads	7173	6788	2251	1548		4215	3138	655	2885	
Observed species	40.3 ± 3.2	43.8 ± 2.0	68.6 ± 1.0	38.8 ± 0.4	**47.9 ± 7.0**	91.0 ± 2.3	72.6 ± 0.7	26.3 ± 0.8	50.1 ± 0.7	**60.0 ± 14.0**
Simpson diversity	0.5 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	**0.7 ± 0.1**	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	**0.8 ± 0.0**
Shannon diversity	1.9 ± 0.0	3.0 ± 0.0	3.5 ± 0.0	2.6 ± 0.0	**2.8 ± 0.3**	3.3 ± 0.0	3.3 ± 0.0	2.8 ± 0.0	2.9 ± 0.0	**3.1 ± 0.1**

Bacterial composition for different samples

Bacterial-phylum composition differed between samples, indicating individual variation for which some samples comprised of more "unassigned" organisms compared to others (Figure 5.3).

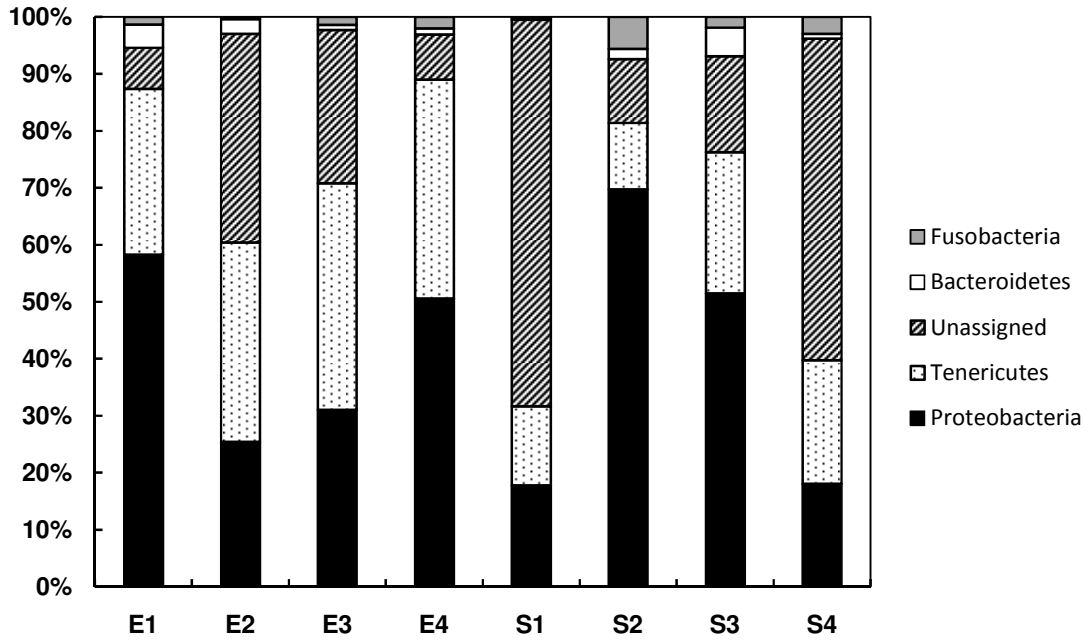

Figure 5.3: Relative abundances of different phyla for each sample for KS diet samples ("E" samples) and control diet samples ("S" samples).

The dominant identified bacterial phyla in all samples were Proteobacteria and Tenericutes, whereas bacteria from Bacteriodetes and Fusobacteria phyla comprised less than 10 % of the gut-bacterial communities in each sample. Many other bacterial phyla (including Actinobacteria, Verrucomicrobia, Planctomycetes, Spirochaetes, Chloroflexi and Firmicutes) collectively comprised less than 1 % of the gut-bacterial communities in abalone gut samples and are therefore not displayed. Identified bacterial genera from the main phyla included *Mycoplasma* (phylum: Tenericutes), *Vibrio* (phylum: gammaproteobacteria), *Polaribacter*

(phylum: Bacteriodetes), *Psychrilyobacter* (phylum: Fusobacteria) and *Shewanella* (phylum: gammaproteobacteria) (Table 5.4).

Table 5.4: Relative abundances (%) are displayed for bacterial classifications at different levels for each sample.

Phylum	Class	Order	Genus	Relative abundance (%)							
				KS diet				Control diet			
				E1	E2	E3	E4	S1	S2	S3	S4
Proteobacteria	δ-proteobacteria	(Uncultured)		38.1	10.4	24.6	37.1	9.2	43.2	29.3	10.2
Tenericutes	Mollicutes	Mycoplasmatales	*Mycoplasma*	28.6	34.7	39.7	38.3	13.8	11.7	24.7	21.6
Unassigned				7.1	36.2	26.9	7.9	67.7	11.2	16.7	56.3
Proteobacteria	γ-proteobacteria	Vibrionales	*Vibrio*	3.5	8.3	2.1	8.4	6.1	15.2	11.2	4.1
Proteobacteria	ε-proteobacteria	Campylobacterales		9.2	1.4	0.3	1.5	0.7	5.5	5.4	0.6
Bacteriodetes	Flavobacteriia	Flavobacteriales	*Polaribacter*	1.3	0.6	0.0	0.2	0.0	0.1	1.7	0.0
Fusobacteria	Fusobacteriia	Fusobacteriales	*Psychrilyobacter*	1.0	0.3	0.3	1.7	0.2	4.8	1.5	1.9
Bacteriodetes	Cytophagia	Cytophagales		0.1	0.2	0.2	0.1	0.1	0.6	1.4	0.0
Proteobacteria	γ-proteobacteria	Alteromonadales	*Shewanella*	1.3	1.4	1.4	1.5	0.4	4.1	1.4	2.1
Proteobacteria	γ-proteobacteria	Cardiobacteriales		0.5	0.6	0.0	0.3	0.2	0.9	1.2	0.3
Bacteriodetes	Flavobacteriia	Flavobacteriales		1.6	0.4	0.3	0.3	0.0	0.1	0.7	0.5

Comparison of abalone gut-bacterial community structure between diets

There was a significant difference in the relative abundance of Tenericutes between abalone fed different diets (Mann-Whitney $U_{4, 4} = 0.0$, $z = 2.17$, $p = 0.03$; Figure 4.4), but, due to individual variation, there were no significant differences in relative abundances of "unassigned" phyla or other gut-bacterial phyla for abalone fed different diets (M-W $U_{4, 4} = 2 - 7$, $z = -1.0 – 1.59$, $p = 0.11 – 0.89$).

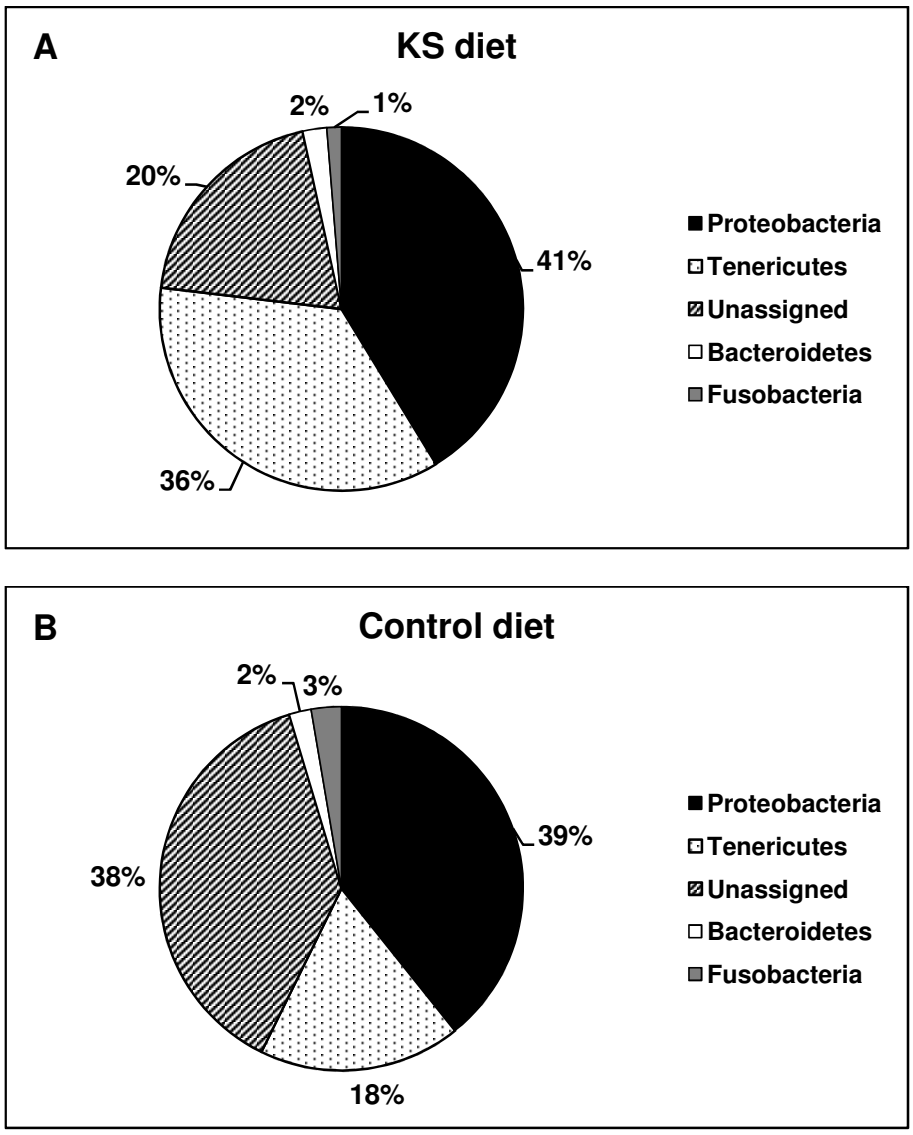

Figure 5.4: The relative abundances (%) of different phyla of gut-bacterial communities for abalone fed the KS diet (A) and the control diet (B).

Overall, there was no difference in the gut-bacterial composition of abalone fed the two different diets as was established with a n-MDS plot (Figure 5.5) and an One-way ANOSIM analysis (R-value = 0.18, p = 0.17). Although the n-MDS plot indicated that KS and control diet samples clustered separately, there was also dissimilarity between samples within the same group as not all the samples within either the KS or control diet group clustered together (Figure 5.5).

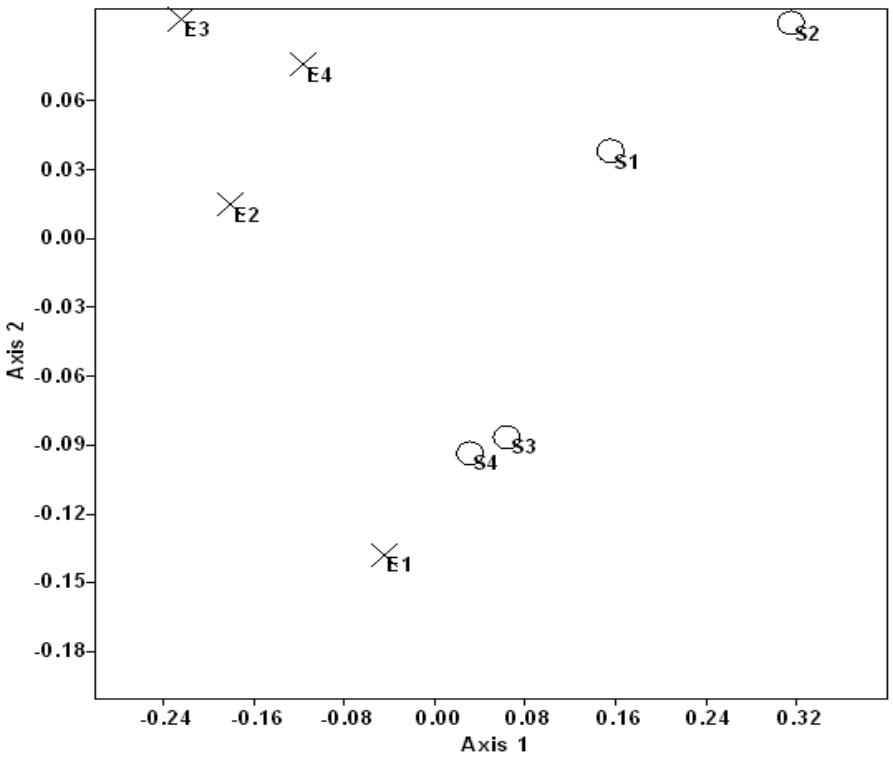

Figure 5.5: A n-MDS plot (stress-value = 0) for samples of the KS diet ("E"-samples) and the control diet ("S"-samples) using a Bray-Curtis measurement of similarity (R^2 values = 0.84 and 0.04 for axis 1 and 2 respectively).

Denaturing gradient gel electrophoresis

Abalone fed KS diets displayed similar band patterns for gut DNA samples, whereas abalone fed the control diet displayed more variable gut DNA band patterns (Figure 5.6). Presence/ absence band data (Table 5.5), indicated dissimilarity between "S" samples of the control diet-fed abalone and "E" samples of the KS diet-fed abalone as was displayed by a n-MDS plot (Figure 5.7).

Table 5.5: DGGE band presence/ absence data used for n-MDS analysis.

S1	S2	S3	S4	S5	S6	S7	E1	E2	E3	E4	E5	E6	E7	E8
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	0	0	1	0	0
0	0	0	1	1	1	1	1	1	0	0	0	0	0	0
0	0	0	1	0	1	1	1	1	1	0	0	0	0	0
0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
1	1	0	0	1	1	0	0	0	0	0	0	0	0	0
1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	0	0	0	0	0	0	0	0	0

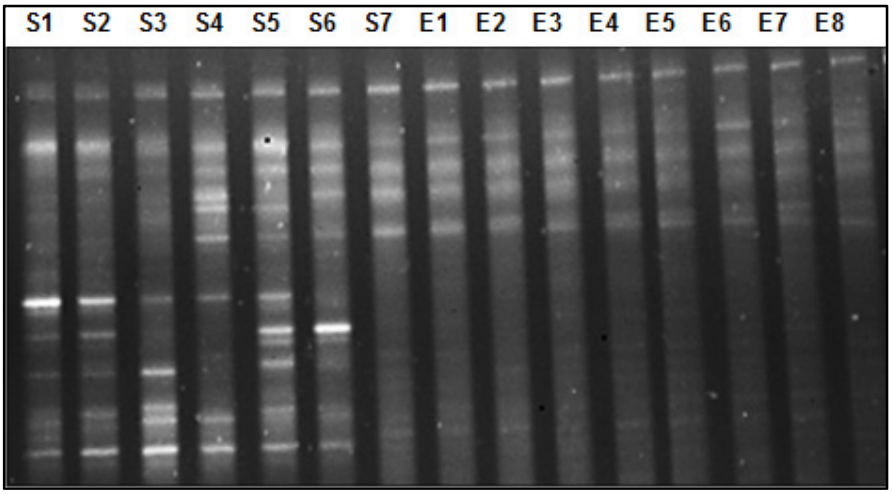

Figure 5.6: DGGE DNA band patterns are displayed for abalone gut samples for control diet samples ("S" samples) and for KS diet samples ("E" samples).

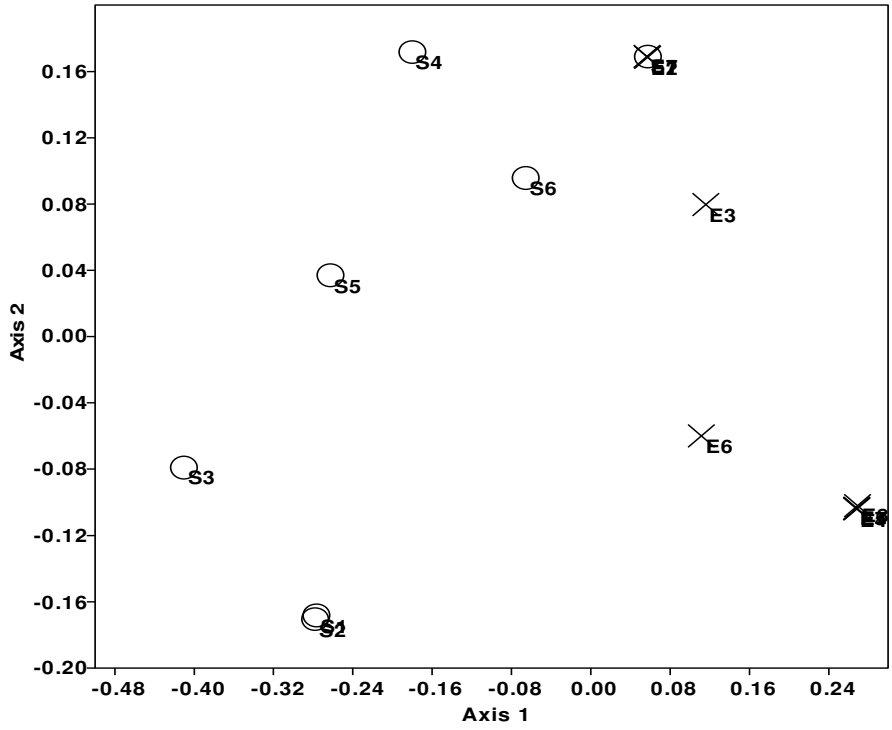

Figure 5.7: A n-MDS plot for DGGE band patterns (stress-value = 0.088) for samples of the KS diet ("E"-samples) and the control diet ("S"-samples) using a Bray-Curtis measurement of similarity (R^2 values = 0.87 and 0.13 for axis 1 and 2 respectively). Greater dissimilarity between S-samples can be observed compared to dissimilarities among E-samples.

DISCUSSION

Gut bacterial community diversity as indicated by the Simpson diversity indices was relatively high for both the kelp-supplemented and control diet fed abalone and were within the same range as values reported for gut bacterial communities for *Haliotis gigantea* abalone fed either formulated feed (1 – D = 0.62) or formulated feed supplemented with a probiotic (1 – D = 0.89) (Iehata et al. 2014). Both Shannon and Simpson diversity indices displayed similar means between diets, but within-group bacterial diversities of individual gut samples for control diet samples were more variable compared to that of KS diet samples. The lower diversity recorded in some of the control diet samples may be attributed to high proportions of "unassigned" organisms that, due to their unknown identity, could not be clustered into different bacterial or prokaryote taxonomic levels.

In this study, Proteobacteria and Tenericutes were the dominant phyla in the abalone gut-bacterial communities and made up about 90 % of all phyla. This finding is in agreement with that of Tanaka et al. (2004) and Huang et al. (2010) whose studies identified Proteobacteria and Tenericutes as the dominant bacterial phyla in the guts and intestines of cultured artificial feed-fed *H. discus hannai* and macroalgal-fed *H. diversicolor,* respectively. Similar to the findings of this study, Huang et al. (2010) identified deltaproteobacteria as the dominant class under the proteobacteria classification, for which the representative bacterial genus was also allocated to an uncultured organism. Macroalgal-fed *H. discus hannai* displayed a greater dominance of Tenericutes compared to what was found for the present study (Tanaka et al 2004).

The class Mollicutes (phylum: Tenericutes) was prominent both in this study and in previous studies on the gut bacteria of abalone (Tanaka et al. 2004; Huang et al. 2010). Tenericutes are phylogenetically related to Firmicutes and *Mycoplasma* is the best characterised genus within the phylum (Madigan et al. 2015). *Mycoplasmas* are small (0.2 –

0.3 μm) coccoid commensal bacteria that live in close association with animal hosts, while being resilient to host immune responses (Razin 2007; Madigan et al. 2015). They utilise carbohydrates as an energy source and require other nutrients needed for growth, such as vitamins and amino acids, from the host (Madigan et al. 2015). *Mycoplasmas* were also prominent in the intestines of farmed and wild salmon in a study that proposed that the commensal role of *Mycoplasmas* in their aquatic hosts may be unknown (Holben et al. 2002).

Bacteriodetes and Fusobacteria were the third and fourth most dominant identified bacterial phyla in this study, but both phyla comprised a smaller percentage (< 10 %) of the abalone gut-bacterial community compared to the proportions of "unassigned" organisms, which also exceeded the two dominant phyla in some samples. The "unassigned" bacteria are most likely a reflection of the limitations of bacterial databases and might fall under the Proteobacteria phylum for species that have not been identified before and would share similarities with known bacteria if a lower threshold for bacterial clustering was selected. Tanaka et al. (2004) identified two bacterial clones that shared less than 90 % sequence similarity to previously sequenced organisms in the database, which indicated that those bacteria were evolutionary divergent from other Proteobacteria (into which phylum they were clustered). Both the Bacteriodetes and Fusobacteria phyla have also been identified in gut samples of other abalone species (Tanaka et al. 2004; Zhao et al. 2012; Iehata et al. 2014).

In the present study, Cytophagia and Flavobacteriia classes that were identified under the Bacteroidetes phylum are chemoorganotrophic degraders of complex organic molecules such as cellulose, chitin and pectin (Reichenbach 2006) and are typically associated with dissolved organic matter in marine environments (Kirchman 2002). Bacteroidetes comprises one of the major phyla of the human gut microbiome due to their characteristic ability to degrade complex molecules such as polysaccharides and proteins (Marchesi 2010). Interestingly, the presence of Fusobacteria in this study only corresponds to the finding of

one other study for which abalone were starved for a week (Tanaka et al. 2004), whereas abalone in the present study were not fed for only one day prior to sampling.

Some of the bacterial genera identified within this study were also identified for abalone gut samples through culture-independent analyses by other studies and include: *Mycoplasma* (Tanaka et al. 2004; Huang et al. 2010; Iehata et al. 2014), *Vibrio* (Tanaka et al. 2004; Huang et al. 2010; Zhao et al. 2012; Iehata et al. 2014) and *Shewanella* (Zhao et al. 2012; Iehata et al. 2014). In addition, pyrosequencing analyses established that the composition of bacterial phyla identified in the stomach of the Eastern oyster *Crassostrea virginica* was similar to that of abalone gut bacteria in the present study even though the relative abundances of bacterial groups were different (King et al. 2012). The stomach microbiome of *C. virginica* within a specific locality displayed a high dominance of *Mycoplasmas* (Mollicutes), and other stable gut and stomach bacterial groups included alphaproteobacteria, Chloroflexi, Planctomycetes, Firmicutes and Verrucomicrobia (King et al. 2012). These latter groups comprised less than 1 % (0.01 – 0.6 %) of the gut bacterial communities in the present study.

In this study, all individual abalone samples shared the same bacterial phyla, but there was considerable individual variation in terms of the relative abundances of bacterial groups which may have masked variation between diets. However, despite relatively small sample sizes, it seemed that Mollicutes were consistently more abundant and comprised less variable proportions in KS diet samples compared to control diet samples. A higher abundance of Mollicutes in the guts of abalone fed the KS diet may have occurred due to less competition by opportunistic bacteria in the guts compared to that of abalone fed control feeds. Due to the lack of potentially harmful characteristics, Mollicutes have been suggested to be natural residents and commensals in the guts of salmon and long-jawed mudsuckers (Holben et al. 2002; Bano et al. 2007). Interestingly, and perhaps similar to the regulatory response in the

present study, an increase in the number of Mollicutes in the abalone gut was found when *H. gigantea* were supplied with probiotics (Iehata et al. 2014).

The electrophoretic separation of PCR amplified DNA indicated differences in the bacterial communities between abalone fed the different diets. The clear DGGE analysis bands, which expressed the more dominant bacterial species, differed between the two diets and were more variable for gut samples of abalone fed the control diet. The DGGE band patterns displayed a similar pattern to that of the pyrosequencing analyses in that the control diet samples displayed more variation in terms of bacterial composition. In contrast, gut samples from abalone fed the KS diet showed greater similarity between samples, which may indicate that gut-bacterial communities are better regulated in abalone fed the KS diet. Since abalone were fed a kelp-containing commercial diet prior to the experiment (see Chapter 3), the introduction of a control diet that lacks the kelp supplement with potential prebiotic-like or antimicrobial properties (see Chapter 1) may have resulted in the invasion of opportunistic bacteria in the guts of the experimental control diet-fed abalone. Therefore, in addition to maintaining the gut microbiome in its former (core) state, kelp supplementation in abalone diets may effectively modulate abalone gut-bacterial communities by enforcing ecological stabilisation through inhibition of the growth of opportunistic bacteria.

CONCLUSIONS

1) Gut bacterial community diversity was relatively high for abalone fed both the kelp-supplemented and control diets.

2) The same bacterial phyla were present in the guts of abalone fed both kelp-supplemented and control diets, but there was considerable individual variation in terms of the relative abundances of phyla, especially for control diet-fed abalone, which may have masked variation between diets.

3) Kelp supplementation in formulated feed did however result in a higher relative abundance of the Tenericutes phylum (class: Mollicutes), which comprised one of the dominant phyla in the guts of abalone fed both diets and was present at relatively constant proportions in the guts of abalone fed the kelp-supplemented diet. Future studies should establish the role of Mollicutes in the guts of abalone, but it is likely that they are commensals that form part of the core gut microbiota.

4) The greater variation in the gut-bacterial community composition of samples of abalone fed the control diet compared to samples of abalone fed the KS diet, suggests that kelp supplementation in abalone feed promotes a more stable abalone gut-bacterial community through effective gut-bacterial modulation.

CHAPTER 6

CONCLUDING DISCUSSION

This present research demonstrated that the inclusion of kelp in a formulated feed had a pronounced positive effect on abalone growth and feed utilisation efficiency and produced differences in the abalone gut microbiome and its function.

The literature review in Chapter 1, which summarised research on seaweed supplementation of formulated abalone diets, noted the observed growth performance benefits cannot be explained by the macronutrient composition of macroalgae alone. It was suggested that gut-bacterial modulation by seaweed probably plays an important, but as yet poorly understood, role in abalone digestion. It was therefore hypothesised that the role of the kelp supplement on the composition of abalone gut-bacterial communities may have contributed to the observed positive effect of dietary kelp inclusion on abalone growth and feed utilisation efficiency on South African commercial abalone farms. The thesis research approach therefore consisted of a series of four investigations designed to find evidence of: 1) the positive effects of kelp supplementation on abalone growth and feed utilisation efficiency; 2) changes in the gut bacterial community profile between abalone fed the kelp-supplemented and control diets; and 3) differences in the abalone digestive tract morphology and 4) enzyme activities between the diet treatments that might be due to bacterial metabolic activity.

The on-farm growth trial in Chapter 2 established that kelp inclusion (0.44 – 3.54 % dry mass) in formulated abalone feed produced faster growth and better FCR and PER values in cultured abalone subject to standard husbandry practices in comparison to the control diet with no kelp. Mass gain (%), FCR and PER were significantly correlated with kelp level in

the range of 0.00 – 3.54 % dry mass. As the control and kelp-supplemented experimental feeds had similar macronutrient compositions, the beneficial effect of the kelp was probably due to unique properties of kelp, such as its probiotic (vector for macroalgae-degrading bacteria), antibacterial or prebiotic properties and/ or its contribution of micronutrients, antioxidants or immuno-modulators, promoting an additional health or nutritional effect.

It was suggested that the kelp supplement may function as a prebiotic which would stimulate the selective growth of beneficial enzyme-producing fermentative bacteria that would increase the production of volatile short-chain fatty acids in the gut, which might in turn act as trophic factors for epithelial cells in the gut, leading to changes in morphology. Both digestive enzyme activity levels and morphological changes associated with potential modifications of short-chain fatty acid levels in the gut were therefore compared between abalone fed the kelp-supplemented and control diets.

Kelp supplementation did not however induce any observable changes in abalone gut morphology (Chapter 3). This may have been due to the similar macronutrient composition of kelp-supplemented and non-supplemented formulated feeds, the sampling time after a relatively short period on experimental diets and the similar diet history of experimental animals on a commercial kelp-supplemented formulated feed prior to the experiment. Their common diet history prior to the seven-week experiment could have shaped gut-bacterial fermentation pathways and crop epithelial cell morphology. Future studies should combine histological investigations in the gut with the levels and types of fatty acids present, and should establish the utilisation of specific fatty acids as trophic factors for cells in different regions of the gut.

Scanning electron microscopy analysis of the crop surface of the abalone fed the experimental diets revealed two types of bacteria associated with the crop epithelium which can be identified with relative certainty. These were a rod-shaped bacterium and a

filamentous bacterium which seemed to occur commonly within the abalone crop and are therefore able to withstand both the relative acidity and the presence of lysozymes in the crop (established in Chapter 4). To characterise the bacteria present in the abalone gut, the next step in the present research was to employ more powerful molecular techniques to identify different types of bacteria and the associated bacterial enzymes in the abalone gut.

The bacterial proteins that were identified by means of proteomic techniques (Chapter 4) indicate that abalone gut-bacterial enzymes play a different and complementary role to abalone endogenous enzymes. While the digestive enzymes secreted by abalone hydrolyse large carbohydrate and protein molecules to their smaller components, the nature of the bacterial proteins indicates that bacterial enzymes probably ferment waste and hydrolysis end-products to new organic compounds. Although the proteins derived from bacteria in the Tenericutes phylum were not identified in this study by using existing databases, bacterial proteins from bacteria that fall under the other dominant phylum, Proteobacteria, were identified. In addition, known bacterial proteins are likely to be conserved across different species with similar metabolic functions. The contribution of bacterial enzymes to carbon transfer in the host (Barry et al. 2002) will need to be established by monitoring the absorption of bacterial-reduced organic compounds by the host through metabolomic studies.

The absence of differences in polysaccharidases and acid proteases in the guts of abalone fed a kelp-supplemented and a non-supplemented diet may be attributed to the fact that enzymes under these groups were mostly endogenous. These enzymes may have displayed similar activity levels due to the relatively similar quality and nutrient composition of the diets. However, the greater variability in digestive enzyme activity levels observed in abalone fed the control diet compared to those fed the kelp-supplemented diet may have been caused by their more opportunistic gut-bacterial communities. It is hypothesised that the control diet produces a variable composition of gut-bacterial communities paired with

variable bacterial metabolic activities which leads to inconsistent bacterial utilisation of the measurable reduced organic end-products of abalone enzyme hydrolysis, compared to the kelp-supplemented diet.

The genomic characterisation of abalone gut-bacterial populations indicated a shift in bacterial composition in terms of the relative abundances of bacteria, both at a higher taxonomic level and for individual species when abalone were fed a kelp-supplemented diet compared to a non-supplemented one. However, greater within-group variation for bacterial composition in the guts of abalone fed the control diet resulted in less well defined bacterial patterns in this group, which would reflect a less ecologically stable gut microbiome in abalone fed the control diet compared to those fed the kelp-supplemented diet. The increase in Mollicutes in abalone fed the kelp-supplemented diet is likely to reflect a restoration of gut-bacterial communities to its more natural state, since the gut bacteria of farmed *H. discus hannai* abalone fed a natural macroalgae diet was dominated by Mollicutes (Tanaka et al. 2004).

The within-group similarity of gut-bacterial composition for individual abalone fed the kelp-supplemented diet, established with both pyrosequencing and DGGE analyses, suggests that the bacterial communities in abalone fed kelp-supplemented feeds are more stable. This also suggests that the kelp supplement in formulated feeds has a selective effect on abalone gut-bacterial communities, which may contribute to animal homeostasis which increases the abalone's ability to withstand variable environmental and water quality conditions on the farm. Interestingly, in agreement with the phenomenon of the present study, a study on weaning piglets found greater similarity of faecal bacterial DGGE band patterns for piglets fed diets with soluble fibre supplementation (fructooligosaccharides and sugar beet pulp < 10 % (w/w)) compared to those fed a control diet, reflecting the ecological stabilisation of gut

bacteria in response to the supplement during a vulnerable growth phase (Konstantinov et al. 2003).

The alternative mechanism for gut-bacterial modulation through low-level kelp supplementation is the introduction of beneficial kelp-associated bacteria in the abalone gut with the ingestion of food. The host-bacteria ingestion association, for which food acts as a vector for bacterial introduction to the gut, leading to subsequent proliferation of bacteria in favourable gut regions, was suggested to be the most common relationship between bacteria and invertebrates (Harris 1993). While this vector relationship to supply additional gut bacteria may be valid for abalone continuously fed fresh seaweed (Kemp et al. 2015), it is unlikely to explain the differences in gut bacteria observed between the dietary treatments in the present study. Kelp-supplemented formulated feed is subjected to extrusion, drying and storage which bacteria are unlikely to survive. In addition, the similar macronutrient composition and high nutrient density of the kelp-supplemented and non-supplemented feeds and the presence of oxygen in the culture tanks are likely to stimulate the growth of similar bacterial communities on feed pellets during their submersion in the water prior to ingestion. Instead, the selective effect of low-level kelp supplementation in the guts of cultured abalone in the present study is more likely obtained with prebiotic-like (selective growth stimulation) or selectively-antibacterial effects. In pigs, dietary supplementation with brown seaweed and it extracts produces an antibacterial effect on gut bacteria in weaned piglets (Reilly et al. 2008; Dierick et al. 2009) and adult pigs (Smith et al. 2011), which is especially effective on opportunistic *E. coli* species and other species of Enterobacteriaceae.

The bacterial-modulating mechanism of the kelp supplement is most likely a bactericidal/ bacteriostatic mechanism or a mechanism that stimulates selective fermentation (prebiotic) and other bacterial metabolic activities. Thus, while probiotic supplementation may be used to artificially increase digestive enzyme activity levels in abalone by

continuously supplying probionts to abalone at relatively high dosages (Macey & Coyne 2005; Macey & Coyne 2006), kelp supplementation could function to promote physiological homeostasis, health, a stable gut microbiome and regulated digestion efficiencies in farmed abalone. In addition, dietary kelp supplementation for farmed abalone may achieve gut-bacterial modulation through a less intensive "natural" approach.

In conclusion, this thesis established that kelp supplementation in formulated feeds results in a more controlled gut microbiome and stable enzyme activities in cultured South African abalone. The stability of the gut microbiota in abalone fed the kelp-supplemented diet may reflect the core commensal gut-bacterial community composition of abalone. In addition, the higher relative abundance of Mollicutes may reflect the restoration of a stable community of commensal Mollicutes which may have been threatened by the presence of opportunistic bacteria in abalone fed the control diets. The role of Mollicutes in abalone digestive physiology and health remains to be established.

The results have shown that kelp can be efficiently used as a dietary supplement in the formulated feeds of cultured abalone to enhance production performance and to manage gastrointestinal health. The implications of managing gut health in cultured abalone can include improved resilience to stress caused by changes in water temperature and water quality in the farm environment since more metabolic energy can become available to counter these effects. A balanced and regulated gut environment and microbiome allows the energetically costly process of digestion to become more efficient and limits the need for the immune system to act against opportunistic bacteria. Kelp is a resource that is available to abalone farmers in the Western Cape and by further optimising its use and inclusion level in formulated feeds by focusing on its effects on the abalone gut environment and immune system, farmers will have a useful, practical and cost-effective tool to manage abalone health and performance.

The common diet history of experimental abalone on the same weaning diet followed by the same kelp-supplemented diet prior to the study could have shaped the gut physiology of the abalone through developmental effects. It is possible that the endogenous enzyme secretion patterns in the experimental abalone may have been established at a younger age in response to previous diets, which would have caused abalone in the present study to have similar digestive enzyme levels. In addition, the formation of a specific gut environment in response to diet for the developing experimental abalone, or the presence of kelp in the diet fed to abalone for several months prior to the study, could have led to the establishment of a core gut microbiome. A similar core gut microbiome for experimental abalone could have caused both the levels of exogenous digestive enzymes and the gut morphology of abalone fed different diets in the study to be similar. Future research should focus on developmental studies to test the effect of artificial feed, kelp-supplemented artificial feed and fresh kelp on the gut bacterial communities, gut morphology and digestive enzymes of post-weaning abalone of different age classes to understand the effect of diet history on abalone physiology and production performance.

The platform is now set for future studies to compare the role of the core gut microbiome in cultured abalone fed formulated feed to that of abalone fed macroalgae and also to that of wild abalone. Studies on the co-evolutionary relationships between abalone and their commensal gut bacteria, for which metabolic roles can be identified with proteomics, may shed further light on the role of gut bacteria in abalone. Commensal gut bacteria are likely to have adapted to the abalone gut to have become specialists with a slow mutation rate (Dr. Bronwyn Kirby, personal communication), and therefore identification of their transcriptome will reveal their association with abalone. The next step would be to establish how opportunistic gut bacteria affect the abalone immune system compared to

commensal gut bacteria, since the latter is likely to have developed means to withstand the abalone immune system (Razin 2007).

The application of proteomics to abalone nutrition is a novel approach and has provided new insight into the functional role of the gut microbiota in abalone digestive physiology. In particular, gut-bacterial enzymes appear to play a complementary role to that of abalone digestive enzymes. Many intricate bacterial metabolic pathways involved in the conversion of organic compounds may be present in the abalone gut. Future studies can further establish the role of gut-bacterial communities on cultured abalone nutrition by quantifying the contribution of gut bacteria to nutrition in abalone through metabolomic studies, which can identify microbial-derived metabolites present in the host gut and plasma (Jacobs et al. 2009). This will identify which bacterial groups play the most important roles in integrating with the abalone hosts' metabolism through the production of compounds that can be used as energy sources or essential nutrients by the abalone host.

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